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THE ACTINOMYCINS AND THEIR IMPORTANCE IN THE TREATMENT OF TUMORS IN ANIMALS AND MAN

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ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

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October 5, 1960

THE ACTINOMYCINS AND THEIR IMPORTANCE IN THE TREATMENT OF TUMORS IN ANIMALS AND MAN*

Conference Chairman and Consulting Editor

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INTRODUCTION

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Just 20 years ago, namely on April 23, 1940, I presented before the National Academy of Sciences, meeting in Washington, D.C., a paper entitled "The Soil as a Source of Microorganisms Antagonistic to Disease-Producing Bacteria." This presentation may be considered an important historical event, since (1) it was, to my knowledge, the first address ever to have been given before the National Academy on the subject of antibiotics, (2) it reported on the formation and isolation of the first true antibiotic produced by actinomycetes, a group of microbes that later came to occupy a leading place as producers of antibiotics, and (3) this antibiotic, designated as actinomycin, was soon to be isolated in crystalline form.

Actinomycin, although it was too toxic to receive serious consideration as a potential chemotherapeutic agent, showed some peculiar effects upon certain organs in experimental animals, particularly its action upon the spleen of the animals. The antibiotic had to wait, however, more than a decade before the significance of its cytostatic properties were recognized.

Actinomycin was extracted in ether from the culture broth of the organism designated *Actinomyces* (now *Streptomyces*) *antibioticus*. The substance was crystallized from alcohol as a red pigment. On chemical analysis it showed the structure of a polycyclic polypeptide. It was characterized by a typical antimicrobial spectrum, with high activity against Gram-positive bacteria (1:100,000,000), limited activity against Gram-negative bacteria, and still less activity against fungi.

Various attempts were made to reduce its toxicity, but such reduction resulted in decreased activity. Because of this, further work on it was practically stopped, and efforts were directed toward the isolation of less toxic antibiotics. During the next decade the subject of antibiotics developed into an important science, with numerous theoretical and practical applications. The actinomycetes themselves proved to be a highly fruitful group of antibiotic-producing organisms.

Actinomycin was not forgotten, however. In addition to the form isolated in 1940 and designated as A, another was isolated by H. Lehr and J. Berger and designated as B. This was later followed by the isolation of a third form, C, by H. Brockmann and N. Grubhofer.

In 1952, a group of German pharmacologists and clinicians demonstrated that actinomycin C had a remarkable effect in repressing the growth of certain tumors, especially those concerned with Hodgkin's disease, or the lymphomas. This effect was seen not only in experimental animals but also in man.

These results attracted immediate and universal attention throughout the world. Some of us went so far as to claim that finally we had discovered a "cure" for the dreadful group of diseases known as cancer. Various laboratories and clinics became interested in the question. Unfortunately, the original claims concerning the effectiveness of the antibiotic were not fully substantiated. Many of the results tended to indicate, however, that actinomycin possesses a definite repressive effect upon certain types of tumor, both in animals and in man. It was found to be very effective in certain tumors and in combination with other methods of treatment in others. The excessive toxicity of the various preparations remained, however, the controlling factor in forewarning against their general use.

Studies carried out at first by Brockmann and his associates and later by others brought out that the various actinomycins differ in their chemical composition. It was established that the actinomycins are made up of several chemical entities and that their structure depends upon the nature of the actinomycin-producing organism and the composition of the medium. A form of actinomycin isolated in our laboratories and designated as actinomycin D appeared to be more homogeneous in chemical composition and to possess a somewhat less toxic effect.

Recently evidence has begun to accumulate indicating that the structure of the actinomycin molecule may be modified by changes in the nature of the medium in which it is produced. There also are indications that in this influencing of the biogenesis of the actinomycin molecule its toxicity also may be modified.

In view of the growing interest in this antibiotic, the accumulated information is presented here and thus made readily available to all who are interested in the study of a highly important group of antibiotics and in their use in clinical medicine.

I take this opportunity to express my sincere appreciation to all my colleagues who have contributed toward the publication of this monograph. If only one additional human life will be saved as a result of these efforts, all this work will have been fully justified.

HISTORICAL BACKGROUND

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Much has been written on the origin of the knowledge of antibiotic action and the organized search for antibiotics. It is clear that in spite of the many early observations the full significance of antibiotics has been realized only within the last 20 years.

Actinomycin was the first crystalline antibiotic derived from a *Streptomyces*.[1] It was isolated and crystallized 20 years ago, in 1940, and was described at a meeting that year of the National Academy of Sciences in Washington, D.C. That this discovery occurred in the Soil Microbiology Laboratory of Rutgers University was no accident. The associative relationships of microorganisms long have been a subject for soil microbiologists and had been under investigation at the laboratory. Three years before the discovery of actinomycin, Waksman and Foster[2] had published a report entitled "Associative and Antagonistic Effects of Microorganisms."

As early as 1931, René Dubos, a former student of the Soil Microbiology Laboratory, ingeniously had applied the familiar soil enrichment techniques to a medical problem and had discovered in soil bacteria an enzyme that removed the capsule of a pneumococcus, making it more available to the host-resistance mechanism of the body.[3] Later, following similar techniques, Dubos and his associates obtained, also from a bacterium, a true antibiotic, tyrothricin, capable of attacking pathogens both *in vitro* and *in vivo*.[4,5] Meanwhile Florey, Chain, Abraham, and their active group at Oxford University, Oxford, England, utilizing Fleming's observation of the antagonistic action of a mold, discovered an antibiotic of great significance: penicillin.[6] The time was ripe for a planned approach to the discovery of a new antibiotic, and such an approach was undertaken at Rutgers University.

That the actinomycetes were emphasized in this approach also was no accident. The Soil Microbiology Laboratory at Rutgers was a center for the study of these interesting microbes. The actinomycetes had become recognized as one of the more numerous groups of the soil flora. Much scientific argument had raged over their significance in the soil, and even in 1940 workers in most bacteriology laboratories would have considered them the least likely to yield interesting products in abundance.

The work on actinomycetes at Rutgers dates back to 1915–1916, when Waksman and Curtis[7] obtained hundreds of these organisms from soils, had become fascinated with their diverse pigments and structures, and laid the groundwork for our present classification system. It is interesting that nearly simultaneously with Waksman and Curtis' basic studies Greig-Smith[8] in Australia developed the theory that actinomycetes control or limit the development of other members of the soil population. This investigator noted the prevalence of actinomycetes that limit bacterial growth on agar plates, and he demonstrated that the white forms were more generally toxic toward bacteria than the chromogenic forms. Even at that time the antagonistic nature of the actinomycetes was recognized widely. The first report of antagonistic action of an actinomycete had been published 27 years earlier, in 1890.[9]

With this background, therefore, the program, which was to culminate in the discovery first of actinomycin and then of a series of important antibiotics from actinomycetes, was begun with the soil as a source of the antibiotic-producing cultures.[10] The organism producing actinomycin was found in a soil that repeatedly had been fed living bacteria until it had become literally an overflowing cemetery for microorganisms. Garden soils were placed in unglazed porcelain crocks at optimal moisture content and were supplemented with living washed cells of *Escherichia coli* at about weekly intervals for four months. The results of such treatment are presented in FIGURE 1. *E. coli* became less able to persist in the soil with each subsequent addition until, finally, after an eleven-day test period, it could not be recovered.

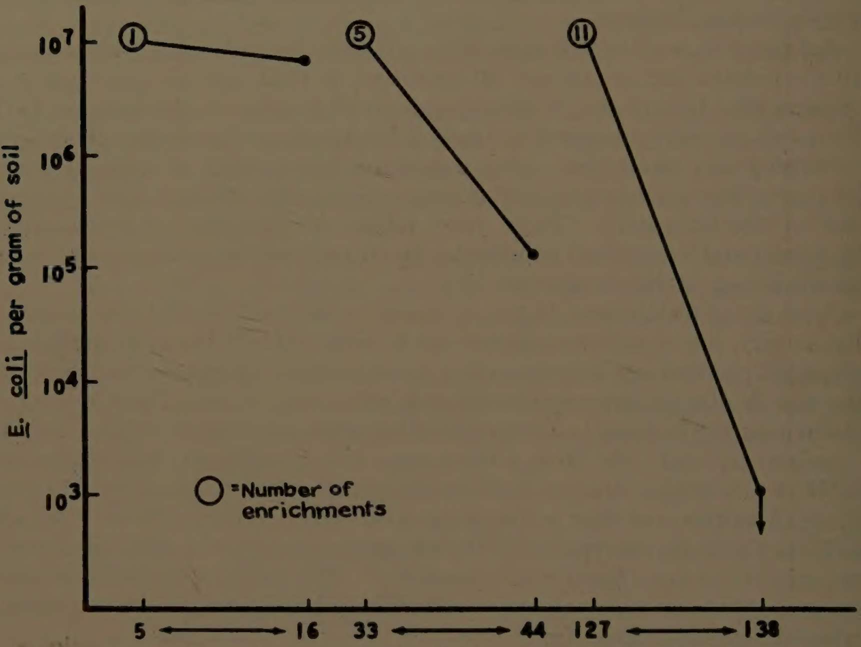


FIGURE 1. Relation of the frequency of addition and survival for 11-day periods of *E coli* in soil.

In spite of the rapid rate at which *E. coli* disappeared in the supplemented soils, the soils were not generally lethal or toxic. The total living population in the soils, as estimated by plating on egg albumen agar, increased during the enrichment (FIGURE 2). This enriched, highly populated soil served as a source of antibiotic-producing microbes.

To reveal antibiotic producers, samples of the soil were diluted in an agar medium containing living cells of *E. coli* as the sole nutrient. Many of the soil microbes grew. Those capable of lysing the living *E. coli* cells gave clear areas, and the colonies in the centers of these clear areas were selected for further study (FIGURE 3). Clear areas due solely to bacteriophage action also were found. Many clear areas surrounded colonies of bacteria; however, the closest attention was given to actinomycetes found within lytic zones.

From such an area an actinomycete was isolated that was different from any other culture previously observed. Therefore its characteristics were recorded and the name *Actinomyces antibioticus* was applied, a name later changed to *Streptomyces antibioticus* to correspond to our modern classification scheme. The typical straight sporophores, arranged in a brushlike cluster, and the background mycelium of *S. antibioticus* are shown in FIGURE 4.

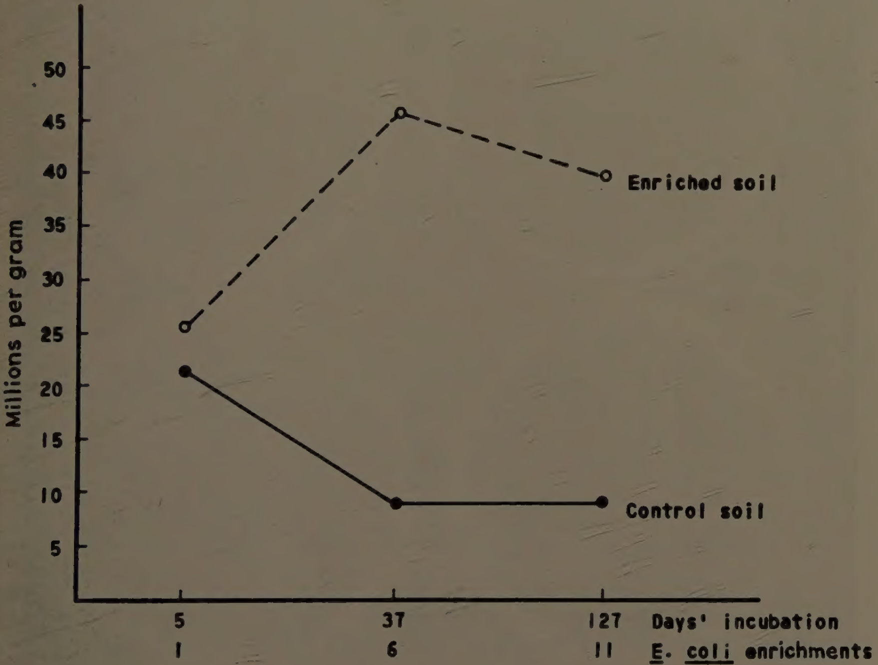


FIGURE 2. Total number of microorganisms in control and *E. coli*-enriched soil.

The detection and isolation of *S. antibioticus* form an example of the importance of chance observation in scientific endeavor. After its isolation, *S. antibioticus* never again was shown to dissolve living cells of *E. coli*. Whether its existence in the original lysis plate resulted from a fortunate combination of a bacteriophage with *S. antibioticus* in the midst of the clear area or from a mixture of an active proteolytic microorganism and the lethal *S. antibioticus*, no one knows. However, the observation did result in the recovery from the soil of a microorganism capable of producing one of the most active of the antibiotics.

To yield actinomycin, *S. antibioticus* was grown on an agar medium containing complex nutrients. The studies had been conducted before the introduction of the shake flask technique for the cultivation of *Streptomyces*. Streptomycetes produce a very thin pellicle on the surface of liquid stationary media. Far better growth is obtained on the surface of a solid substrate such as agar.

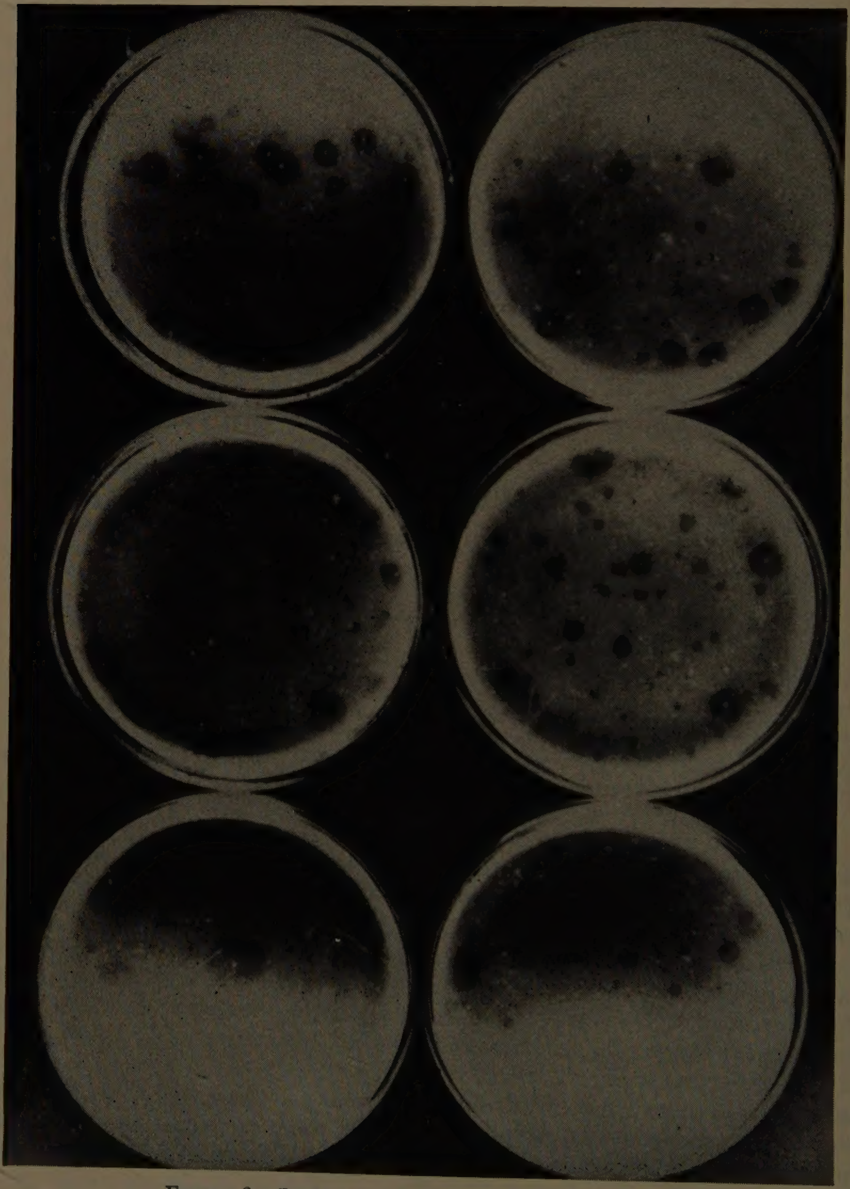


FIGURE 3. Lytic microorganisms in an enriched soil.

Water extracts were made of agar cultures and these were found to be strongly inhibitory to the growth of many microorganisms.

Purification of actinomycin was facilitated by its solvent solubility. Either Soxhlet or batchwise extraction with ether removed the antibiotic from the agar culture. Actinomycin is insoluble in petroleum ether but soluble in alcohol and in most other organic solvents. Crystallization of the antibiotic was readily achieved by treatment with solvents and evaporation. Actinomycin A was a red-pigmented substance highly active against Gram-positive bacteria and, to a lesser degree, against Gram-negative microorganisms. A typical spectrum is shown in TABLE 1. To experimental animals the antibiotic was highly toxic, doses of 0.5 to 1.0 mg./kg. of body weight being lethal. The most striking pharmacological effect of actinomycin was upon the spleen, which was reduced in weight and size from one half to one tenth the original. At nontoxic levels actinomycin had no therapeutic effect on bacterial infections in animals.[11]

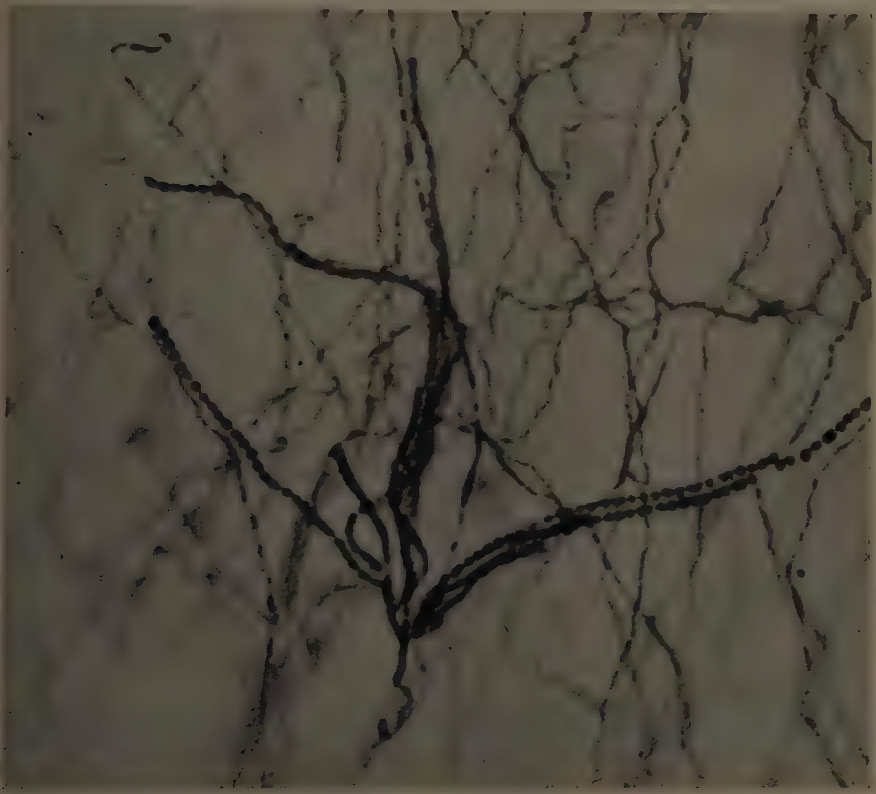


FIGURE 4. *S. antibioticus.*

The chemical nature of actinomycin was studied by Waksman and Tishler,[12] who reported it to be a polycyclic nitrogen compound with characteristic absorption in the visible and ultraviolet regions.

Actinomycin has proved to be a commonly occurring antibiotic product of soil streptomycetes. As extensive screening programs were developed, actinomycin was found repeatedly throughout the world to be produced from microbes with different characteristics. Its high toxicity usually was responsible for its discard from such screening programs as soon as its identity was established.

Since the original description of *S. antibioticus* in 1941[13] at least 11 different species names have been applied to actinomycin-producing cultures. As differences in the chemical properties of the actinomycins have become apparent, various letter designations have been given to the actinomycins derived from each culture. The first published report of each new culture or actinomycin complex is presented in TABLE 2. This incomplete listing shows how complex the situation has become, in respect to both names of the producing microorganisms and names of the antibiotic products. Waksman *et al.*[32] considerably clarified the problem of culture nomenclature by showing that the actinomycin-producing strains were of three basic types: chromogenic with straight sporophores, nonchromogenic with straight sporophores, and nonchromogenic with coiled sporophores. Various species names have been applied within the three groups (FIGURE 5).

TABLE 1
ANTIBACTERIAL SPECTRUM OF ACTINOMYCIN

Gram-negative bacteria	Inhibitory concentration µg./ml.	Gram-positive bacteria	Inhibitory concentration µg./ml.
Escherichia coli	100	*Clostridium welchii*	0.6
Pseudomonas aeruginosa	60	*Bacillus megaterium*	0.6
Brucella abortus	60	*Bacillus subtilis*	0.06
Neisseria catarrhalis	30	*Micrococcus lysodeikticus*	0.03
Shigella gallinarum	30	*Sarcina lutea*	0.01
Hemophilus pertussis	6		

Biologists and chemists often disregard the names of microorganisms suggested by authors of papers on antibiotics because of the tendency to split species into subspecies and strains according to minor characteristics. Other authors in reports on antibiotics have grouped microorganisms to an unwarranted extent; for example, the ability to produce a specific antibiotic has been proposed as the primary characteristic for establishing a species. The very valuable studies of Pridham *et al.*[33] have shown clearly that actinomycin is produced by representatives of several of the major groups of the genus *Streptomyces*, crossing not only species lines but subgeneric or sectional lines. TABLE 3, abstracted from their listing, shows the range in morphologic characteristics and pigmentation of the *Streptomyces* reported to produce actinomycins. The original paper should be consulted for references.

Following the clarification of the taxonomical situation provided by the above-mentioned publications, the nomenclature of the actinomycins has been refined. The crystalline preparations of actinomycins recovered from fermentation broths are now recognized to be mixtures of various chemical entities in differing proportions. The variety of different entities is clear from TABLE 4, in which the various actinomycins are identified by subscripts appended to the letter designation for each complex. Most of the entities have been recognized

TABLE 2

CHRONOLOGICAL LIST OF MICROORGANISMS REPORTED TO PRODUCE ACTINOMYCINS

Year	Reference	Name	Actinomycin complex
1941	1	*S. antibioticus*	A
1946	14	Nonchromogenic species	A
1947	15	*S. flavus*	A (J)
1948	16	*S. parvus*	A
	17	*S. flavovirens*	—
1949	18	*Streptomyces* sp.	B
	19	*S. chrysomallus*	C
1951	20	*S. flaveolus*	A (J)
	21	*Micromonospora globosa*	—
1952	22, 23	*Streptomyces* sp.	X
1954	24, 25	*S. flavus*	X (B)
	24, 25	*S. flavus*	I
	24, 25	*S. antibioticus*	I
	24, 25	*S. flavus-parvus*	X (B)
	26	*S. parvullus*	D
	27	*S. cellulosae*	—
	28	*S. michiganensis*	X
	29	*S. antibioticus*	M
1956	30	*Streptomyces* sp.	E
	30	*Streptomyces* sp.	F
1958	31	*S. fradiae*	Z, X

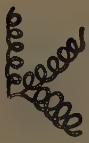
Soluble pigment	Sporophores	Group name	Type species	Complex
Chromogenic	Straight, brushlike	*S. antibioticus*	*S. antibioticus*	A
Nonchromogenic	Straight	*S. flavus*	*S. chrysomallus*	C
	Spiral	*S. parvus*	*S. parvullus*	D

FIGURE 5. Basic classification of actinomycin-producing streptomycetes.

only by their properties as shown on paper chromatography. However, several of the entities have been obtained pure, and it has become apparent that there are duplications among the letter and subscript designations. Because the properties of each actinomycin entity differ, the properties of various mixtures of these entities also differ. When one considers only each pure entity as warranting a name, the situation is clarified.

TABLE 3

SECTIONAL AND SERIES GROUPING OF *STREPTOMYCES* THAT PRODUCE ACTINOMYCIN

Section	Series	Culture	Actinomycin
Rectus-flexibilis*	Olive-buff	*S. flavus*	X (B)
	Yellow	*S. parvus*	A
		S. flavus	I
		S. parvus	I
		Streptomyces sp.	A
		Streptomyces sp.	X
	Gray	*S. antibioticus*	A & B
		S. antibioticus	A
		S. antibioticus (*flavus*)	A
		S. antibioticus	I
		Streptomyces sp.	—
		Streptomyces sp.	B
Retinaculum-apertum*	Gray	*S. flavus-parvus*	B (X)
Spira* 	Red	*Streptomyces* sp.	—
		Streptomyces sp.	A
		Streptomyces sp.	X
	Gray	*S. antibioticus*	—
		S. flaveolus	A (J)
		S. flavus	A
		S. parvullus	D
		S. parvus	—
		Streptomyces sp.	—
		Streptomyces sp.	X
Unknown	White	*S. chrysomallus*	C_1, C_2, C_3
	Yellow	*Streptomyces* sp.	C
	Gray	*S. flavovirens*	—
		S. parvus	—
		Streptomyces sp.	—
		Streptomyces sp.	X
	Unknown	*S. antibioticus*	A
		S. antibioticus	C
		S. cellulosae	—
		S. parvus	—
		Streptomyces sp.	—
		Streptomyces sp.	B
		Streptomyces sp.	B (X)

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A system of nomenclature proposed by Waksman *et al.*[34] is shown in TABLE 5. In this system, Roman numerals are used to designate the various pure entities of the actinomycin complex. Numbers are given only to those actinomycins of which the chemical composition has been determined. Among the actinomycins studied thus far, the structural variations are limited to substitutions among the amino acids of the peptide chains. Certain amino acids in the culture medium can direct the biosynthesis of the microorganism toward the production of new actinomycins.[30,35] In addition, the synthesis of the various actinomycins changes from time to time during the course of a normal fermentation.[36] It is clear, therefore, that names applied to the total products obtained from a fermentation broth have little significance, and that specific names should be reserved for the pure components.

TABLE 4

NAMES ASSIGNED TO VARIOUS ACTINOMYCIN COMPLEXES

Name of complex	Components reported in complex
A	A_{I} A_{II} A_{III} A_{IV} A_{V} A_{VI}
J	
B	B_1 B_2 B_{I} B_{II} B_{III} B_{IV} B_{V} B_{VI}
C	C_0 C_{0a} C_1 C_2 C_3 C_4
X	X_0 X_{0a} $X_{0\beta}$ X_1 X_{1a} X_2 X_3 X_4
I	I_0 I_{0a} I_1 I_2 I_3
D	D_{I} D_{II} D_{III} D_{IV} D_{V}
M	
Z	Z_0 Z_1 Z_2 Z_3 Z_4 Z_5

TABLE 5

PROPOSED TERMINOLOGY FOR PURE ACTINOMYCINS

Basic components
- *N*-Methyl-L-valine (2 moles)
- Sarcosine (2 moles)
- L-Threonine (2 moles)
- 2-Amino-4,6-dimethyl-3-oxophenozazine 1,9-dicarboxylic acid (1 mole)

Variable components
- L-Proline, L-hydroxyproline, 2 D-valine = I
- 2 Sarcosine, 2 D-valine = II
- L-Proline, sarcosine, 2 D-valine = III
- 2 L-Proline, 2 D-valine = IV
- L-Proline, L-ketoproline, 2 D-valine = V
- 2 L-Proline, D-valine, D-alloisoleucine = VI
- 2 L-Proline, 2 D-alloisoleucine = VII

Actinomycin would have died as a complex and interesting, but useless, laboratory curiosity had it not been for the persistence and interest of biologists in their search for agents capable of selectively inhibiting neoplasms. The antibiotics were prime candidates in this search because they are selective in their action on microorganisms; they could be expected, therefore, to be selective in their toxic action on various animal tissues. In fact, selective toxicity of actinomycin was observed, as mentioned before, in its action on the spleen and other organs of the animal body. However, the recognition of actinomycin as a potential antitumor agent came slowly. As early as 1950, in a summary of numerous preparations effective in experimental cancer chemotherapy, Stock[37] reported that actinomycin was the only streptomycete-produced antibiotic that gave detectable levels of inhibition. Subsequently Reilly *et al.*[38] reported that only 5 of 33 known antibiotics, of which actinomycin was one, had a retarding effect upon the growth of Sarcoma 180.

The turning point in the studies came in 1952, when Hackmann[39] described a direct action of actinomycin C, isolated by Brockmann,[19] on Ehrlich carcinoma following contact *in vitro* for 3 hours at 0° C. The tumors thus treated were reduced greatly in their capacity to initiate fresh growth. The antibiotic was effective at dilutions of hundreds of thousands to one, to millions to one. In animals infected with Walker carcinoma, treatment with 50 μg./kg. body weight administered intravenously, orally, or subcutaneously considerably reduced or almost suppressed the development of the carcinoma. Later Hackmann[40] reported that actinomycin C had a cytostatic effect, especially in the lymphatic system. Its therapeutic use in cases of malignant diseases of the lymphatic system was suggested. On the basis of the foregoing studies, Schulte[41] and Schulte and Linge[42] employed actinomycin C as a therapeutic agent in humans.

Clinical developments since these early observations of the cytostatic action of actinomycin have been great. It would not be appropriate to list them here or to attempt to assess the significance of the progress that has been made. The truly significant events in the field will become apparent from the reports comprising the remainder of this monograph.

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Part I. Microbiological and Chemical

THE PRODUCTION OF ACTINOMYCINS BY CONTROLLED BIOSYNTHESES: THE F ACTINOMYCINS

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The red actinomycins formed by certain species of *Streptomyces* and having antibiotic and cytostatic potencies are mixtures of very closely related components, whose peptide chains differ in structure. The actinomycin C mixture isolated from *S. chrysomallus*, according to Brockmann,[1] consists of two main components, C_2 and C_3, and of a by-component, C_1. It may be supposed that actinomycins develop in the cell by oxidative condensation of two *N*-(2-amino-3-hydroxy-4-methyl)-peptide lactones. The actinomycin C-producer, *S. chrysomallus*, forms two such precursors. These have in common the amino acids threonine, proline, sarcosine, and *N*-methylvaline, and differ in that one of them contains valine as a fifth amino acid and the other, alloisoleucine. Oxidative condensation of two valine-containing precursors yields actinomycin C_1; of two precursors containing alloisoleucine, actinomycin C_3; and of one precursor containing valine and one containing isoleucine, actinomycin C_2 (FIGURE 1).

The three actinomycins C and also the components of other mixtures have approximately the same toxicity. For actinomycins of lower toxicity or greater cytostatic potency, attempts were made in 1953 to influence the structure of the peptide chain by the addition of certain amino acids to the medium of actinomycin-producing *Streptomyces*.[2-5] The present report gives an example of such controlled biosynthesis and deals with effect of the amino acid sarcosine on the actinomycin synthesis effected by the *Streptomyces* strain BoP 476, NRRL 2580.

BoP 476 on a synthetic medium forms the actinomycin C mixture. On the addition of sarcosine it produced a very complex mixture of actinomycins. This mixture, termed A 280, separated into three zones on chromatographic partition to alumina. Fractionation, elution, and paper chromatography showed that each zone contained a number of components (FIGURE 2).

Zone 1 contained four components: actinomycins C_1 plus F_5, C_2, and C_3; Zone 2 contained three components: actinomycins F_0, F_2, and F_4; and Zone 3 contained five components: actinomycins F_0, F_1, F_2, F_3, and F_4. The natural actinomycins C_1, C_2, and C_3 were in Zone 1. The three zones of the alumina column were separated into their components by partition chromatography to cellulose powder, with the use of a system of di-*n*-butyl ether, di-*n*-propyl ether, butanol, and an aqueous solution of sodium *m*-cresotinate. The components were crystallized from ethyl acetate/petrol ether.

All actinomycins of the A 280 mixture contained the amino acids threonine, sarcosine, and *N*-methylvaline; actinomycins C_1 and F_0 also contained valine and proline; actinomycins C_2, F_2, and F_5 contained valine, isoleucine, and proline; actinomycin F_1 contained valine and isoleucine but no proline;

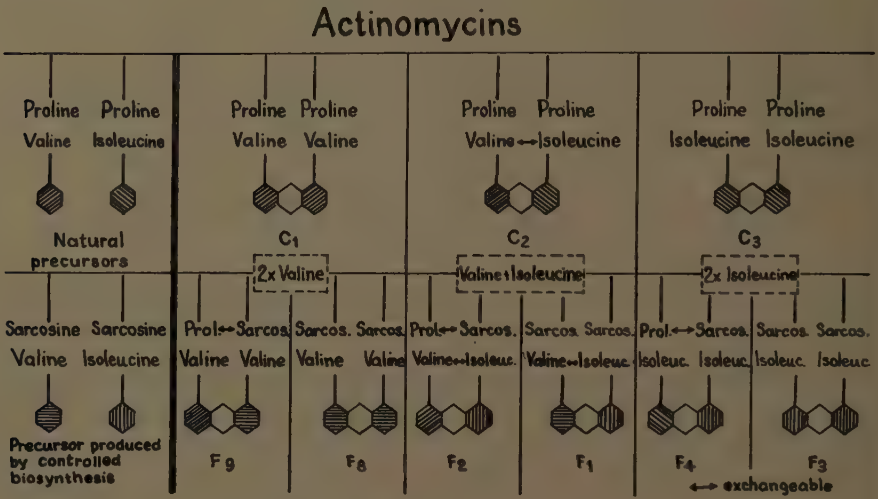

FIGURE 1.

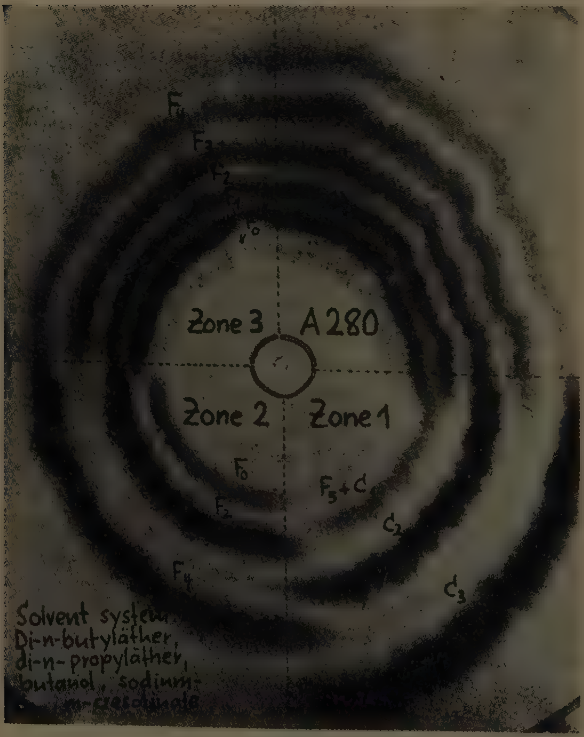

FIGURE 2.

and actinomycin F_3 contained isoleucine but, like F_1, no proline. The components of the mixture A 280 thus differed in their valine, isoleucine, and proline content. It may be inferred that the two precursors naturally formed by *Streptomyces* BoP 476 are modified by the addition of sarcosine in such a manner that sarcosine is substituted for proline in their peptide chains. Two natural precursors and two formed by controlled biosynthesis thus are available to the cell for the synthesis of actinomycins.

The oxidative condensation of a natural valine-containing precursor with one produced by controlled biosynthesis or the oxidative condensation of two formed by controlled biosynthesis should yield actinomycins derived from actinomycin C_1.

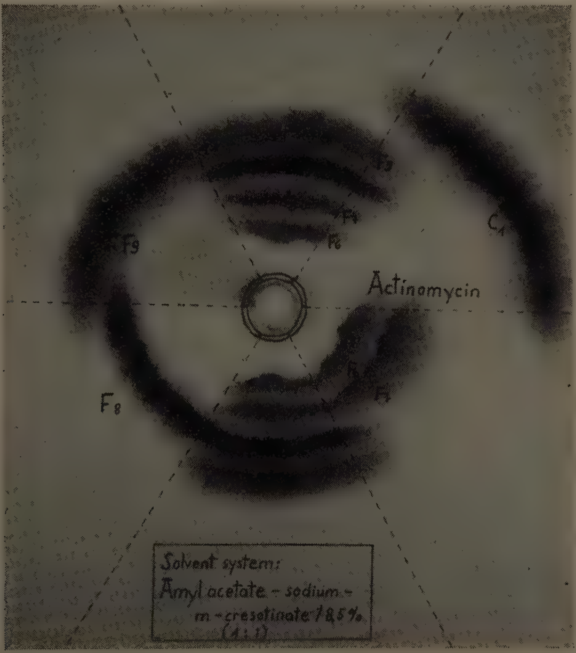

FIGURE 3.

Actinomycin C_1 contains two molecules of valine and no isoleucine. It is the by-component of the actinomycin C mixture and therefore the derivatives of C_1, produced by controlled biosynthesis, can be present in the A 280 mixture only in small quantity. Since the actinomycins produced by controlled biosynthesis have lower R_f values than the corresponding natural products, these actinomycins should appear in the chromatogram within the zone of actinomycin C_1.

Zone 3 of the alumina column contained a small zone with a lower R_f value than that of actinomycin C_1; this is now called actinomycin "F_0". Paper chromatographic analysis showed that it consisted of four actinomycin components, termed, according to their positions on the paper chromatogram from the inside to the outside, F_6, F_7, F_8 and F_9 (FIGURE 3).

These four components could be separated partly by partition chromatography to cellulose powder; the pure components F_8 and F_9 and a mixture of F_6 and F_7 were obtained. Their portion in the A 280 mixture could be estimated as amounting to about 0.1 to 0.5 per cent. The components F_8 and F_9 showed typical actinomycin spectra in the ultraviolet, visible, and infrared ranges (F_8 : $\lambda_{\max} = 449$ mμ, $\alpha = 18.3$; F_9 : $\lambda_{\max} = 446$ mμ, $\alpha = 18.2$; both in ethanol). Their efficacy against *Bacillus subtilis* was determined by plate cup tests as being 38 per cent for actinomycin F_8 and 39 per cent for actinomycin F_9 (actinomycin C_2 = 100 per cent). The hydrolysates of F_8 and F_9 contained threonine, valine, sarcosine, and *N*-methylvaline, but no isoleucine (FIGURE 4). Actinomycin F_9 also had a proline molecule, but F_8 contained none. F_8 and F_9 were derived, as described above, from actinomycin C_1 by the substitution of sarcosine for one or two molecules of proline. The three components, actinomycins C_1, F_8, and F_9, contained valine but no isoleucine.

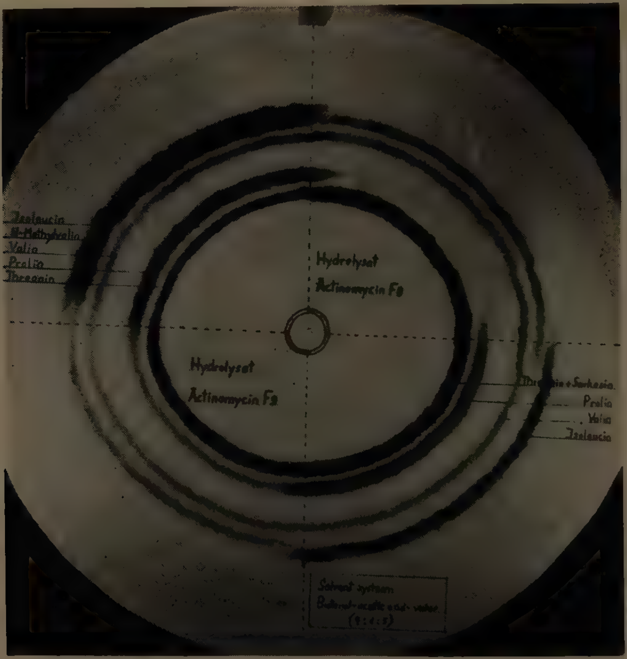

FIGURE 4.

Katz and Goss[6] reported in 1959 two new actinomycin components that had been formed after the addition of sarcosine to the medium of the actinomycin X-producer *S. antibioticus* strain 3720. These new components, which they termed actinomycins II and III, contained threonine, valine, sarcosine, and *N*-methylvaline; component III also contained one molecule of proline, II no proline. The amino acid composition of actinomycin II corresponds to that of actinomycin F_8, while that of III corresponds to that of F_9. It may be that component II is identical with F_8, component III with F_9.

From the hydrolysate of the actinomycin mixture F_6 and F_7 were recovered threonine, proline, sarcosine, *N*-methylvaline, isoleucine, and another amino acid. This further amino acid, which had not before been observed in actinomycins, is identical with glycocoll, according to several systems of paper chromatographic analysis.

Summary

The example of a controlled biosynthesis reported here shows that many new components may be obtained when an amino acid is added to a medium. The addition of sarcosine to the medium of *Streptomyces* BoP 476 yielded 12 actinomycins. Provided the strain forms with sarcosine four precursors, synthesis by oxidative condensation of as many as 16 actinomycins is possible.

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BIOGENESIS OF THE ACTINOMYCINS*

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I would like to indicate a number of areas whose investigation appears important to an understanding of the mechanism of actinomycin biosynthesis. The following list includes many problems that are common to the biosynthesis of other polypeptide antibiotics and some that are related only to the biogenesis of the actinomycin molecule (FIGURE 1):

(1) Relationship of polypeptide formation to protein synthesis.

(2) Relationship of actinomycin formation to the synthesis of such structurally similar polypeptide antibiotics as echinomycin (Keller-Schierlein *et al.*, 1959) and etamycin (Sheehan *et al.*, 1957; Arnold *et al.*, 1958).

(3) Mechanism of lactone formation and time of its occurrence during synthesis of an actinomycin molecule.

(4) Biogenesis of the amino acids D-valine and D-alloisoleucine.

(5) Simultaneous synthesis of several closely related antibiotics by an organism, each differing only at one amino acid site. Usually one component of a mixture is synthesized in major amounts (Goss and Katz, 1957). What determines the extent to which each of the components is synthesized? Can this controlling mechanism be modified?

(6) Derivation of the actinomycin chromophore, 2-amino-4,6-dimethyl-3-phenoxazinone-1,9-dicarboxylic acid. Is tryptophan, 6-methyltryptophan, or acetate the precursor?

(7) Biochemical origin of the methyl groups of the chromophore, sarcosine and *N*-methyl-L-valine. Are they derived from methionine, from formate?

(8) Mechanism of linking the peptides to the chromophore.

It has not been possible for my associates and me to investigate all these aspects of actinomycin biosynthesis; moreover, those that have been studied are still being explored. This paper is a summary of our findings thus far.

Controlled Biosynthesis of the Actinomycins

Brockmann and Pfennig (1953), Schmidt-Kastner (1956*a*,*b*), Goss and Katz (1956), and others have noted that the composition of an actinomycin mixture, synthesized by a given organism, varied somewhat according to conditions of cultivation; and that, in particular, the nitrogen source supplied could influence it greatly, both quantitatively and qualitatively. Thus, Schmidt-Kastner (1956*b*) showed that actinomycin IV increased from 10 to 83 per cent of a mixture synthesized by *Streptomyces chrysomallus* when DL-valine was added to a chemically defined medium. Moreover, he observed that new biosynthetic actinomycins were formed when sarcosine or DL-isoleucine was supplied to this or other actinomycin-producing organisms (1956*a*,*b*).

We had observed quantitative variations in the composition of an actinomycin mixture when different amino acids served as sole nitrogen sources

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(Goss and Katz, 1957; Katz *et al.*, 1958). As the effects usually were limited and there appeared to be neither a chemical nor a biochemical relationship between the amino acid used and the change observed, we assumed that the nitrogen compound influenced chiefly the growth and metabolism of the organism and only indirectly antibiotic synthesis.

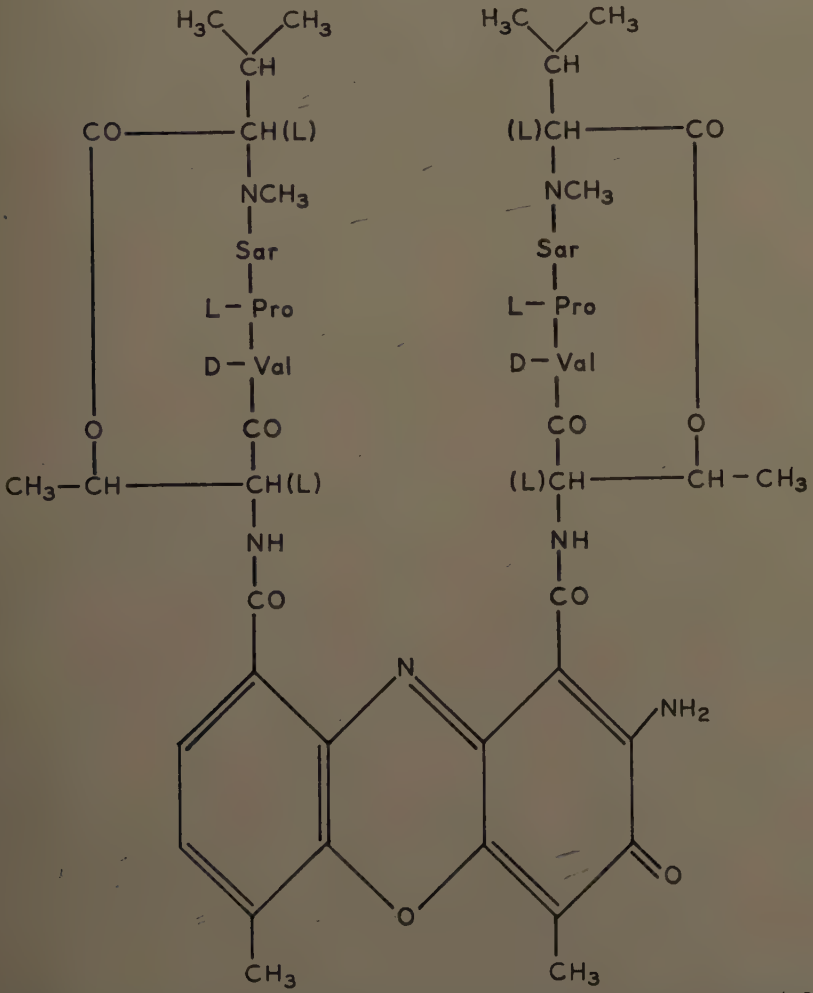

FIGURE 1. Actinomycin IV: The sequence of amino acids is L-threonine, D-valine (D-Val), L-proline (L-Pro), sarcosine (Sar), *N*-methyl-L-valine (Brockmann *et al.*, 1956*c*; Bullock and Johnson, 1957). Actinomycin I: 1 mole of proline is replaced by 1 mole of hydroxyproline (Brockmann *et al.*, 1959). Actinomycin II: 2 moles of proline are replaced by 2 moles of sarcosine (Johnson and Mauger, 1959). Actinomycin III: 1 mole of proline is replaced by 1 mole of sarcosine (Johnson and Mauger, 1959). Actinomycin V: 1 mole of proline is replaced by 1 mole of 4-oxoproline (Brockmann and Manegold, 1958). Actinomycin VI: 1 mole of D-valine is replaced by 1 mole of D-alloisoleucine (Brockmann *et al.*, 1956*c*). Actinomycin VII: 2 moles of D-valine are replaced by 2 moles of D-alloisoleucine (Brockmann *et al.*, 1956). Reproduced by permission of the *Biochemical Journal*.

We decided that, for a directed or controlled synthesis of actinomycin, it would be necessary to employ two nitrogen compounds in the culture medium. The one, present at the time of inoculation of the organism, would serve as the source for protein synthesis and growth, and the other, added at the time antibiotic production began, would direct or moderate the antibiotic synthesis. The amino acids that might serve most effectively as moderators, we thought, would be those at the "variable" amino acid site in the actinomycin molecule (such as hydroxyproline or valine) or those quite closely related chemically to the amino acids in an actinomycin molecule. It was reasoned that these exogenously supplied compounds might compete with the endogenously synthesized amino acids and either (1) be incorporated into the actinomycin peptides with increased synthesis of trace components or formation of new actinomycins or (2) inhibit actinomycin formation by blocking incorporation of the endogenous amino acid.

TABLE 1
INFLUENCE OF HYDROXY-L-PROLINE CONCENTRATION ON ACTINOMYCIN FORMATION BY *S. ANTIBIOTICUS**

Hydroxy-L-proline (μg./ml.)	Actinomycin components (%)					
	I	II	III	IV	V	†
0	7.2	2.9	3.6	59.4	23.2	3.6
100	9.6	3.2	5.5	59.0	19.3	3.4
500	13.4	3.5	9.6	49.0	21.2	3.3
1000	22.5	2.8	7.7	36.9	27.1	2.9
2500	31.0	3.8	7.1	25.3	30.0	3.0

* Hydroxy-L-proline was added to glutamic acid medium after 24-hour incubation of *S. antibioticus*. After an additional 2 days' cultivation the synthesized actinomycin mixture was harvested by extraction with butan-1-ol. Upon removal of the butanol the actinomycin mixture was separated by means of circular paper chromatography and the percentage of actinomycin components in a mixture was determined by a spectrophotometric method (Goss and Katz, 1957).

† Represents a trace component which moves faster than actinomycin V.

The compound first observed to have a direct influence upon actinomycin formation was the amino acid hydroxy-L-proline (TABLE 1). When this was added to the glutamic acid medium, actinomycin I increased from about 6 or 7 to 31 per cent, and actinomycin IV decreased from 60 to 25 per cent, of the mixture synthesized by *S. antibioticus* (Katz and Goss, 1958). We believe this represents a shift from synthesis of actinomycin IV (which contains two moles of proline) to greater formation of actinomycin I (which contains one mole each of L-proline and hydroxy-L-proline).

A more striking effect was found with sarcosine (TABLE 2, FIGURE 2). Actinomycins II and III, produced in trace amounts by *S. antibioticus* in the absence of sarcosine, increased to about 60 per cent of the mixture in its presence (Katz and Goss, 1958, 1959). It is interesting to note that within a day of the sarcosine addition, actinomycins II and III represented 40 to 50 per cent of the mixture synthesized. The effect of sarcosine appears to be quite specific, since a compound biochemically or structurally related—for example, phenylsarcosine, choline, betaine, dimethylglycine, sarcosine anhydride, *N*-acetylglycine, *N*-methylalanine, glycine, or serine—when used instead of sarcosine, essentially was without effect on the synthesis of actinomycins II and III.

When the production of these two antibiotics increased, the synthesis of actinomycin IV greatly decreased. Because actinomycin IV contains two moles of proline, we concluded that the exogenous sarcosine probably competes with endogenously formed proline and is incorporated in place of proline in certain actinomycin peptides and that, as a result, actinomycins II and III are formed at the expense of actinomycin IV. Further studies have provided evidence that this hypothesis is correct.

In an experiment in which a given amount of sarcosine and increasing amounts of L-proline were used we found that the sarcosine effect was appreciably reduced (TABLE 3). Relatively small amounts of actinomycins II and III were formed in the presence of the added proline. When somewhat more sarcosine and the same levels of proline as used before were employed, we were able to reverse the sarcosine effect only slightly.

TABLE 2
INFLUENCE OF SARCOSINE CONCENTRATION ON ACTINOMYCIN BIOSYNTHESIS BY *S. ANTIBIOTICUS**

Sarcosine (μg./ml.)	Actinomycin components (%)				
	I	II	III	IV	V
0.0	6.2	2.1	2.8	80.4	8.4
2.5	6.5	1.9	7.3	70.8	13.6
10.0	5.3	9.2	11.9	63.5	10.1
50.0	6.5	21.2	30.8	35.9	5.6
100.0	8.1	22.5	33.1	30.8	5.5
250.0	8.9	25.4	35.3	24.6	5.7
500.0	9.1	25.6	35.0	24.4	5.9

* Sarcosine was added to the glutamic acid medium after 30-hour incubation of *S. antibioticus*. After an additional 6 days' cultivation, the synthesized actinomycin mixture was harvested by extraction with butan-1-ol. Upon removal of the butanol the actinomycin mixture was separated by means of circular paper chromatography, and the percentage of actinomycin components in a mixture was determined. Reproduced by permission from the *Biochemical Journal.*

Further evidence has been obtained with the proline analogues piperidine-2-carboxylic acid (Zacharius *et al.*, 1954) and azetidine-2-carboxylic acid (Fowden and Bryant, 1958): when the former analogue, pipecolic acid, was added to the glutamic acid medium, as many as five new actinomycins were synthesized by *S. antibioticus* (FIGURE 3); when the latter was used, two to three new actinomycins were formed (FIGURE 4). We also have observed the synthesis of new actinomycins with these analogues when *S. chrysomallus* was employed.

Because of the great similarity between proline and pipecolic acid, it appeared likely to us that the latter compound would compete more favorably with endogenous proline than would sarcosine for the "proline" sites in an actinomycin molecule. The results of an experiment confirmed this supposition. With simultaneous additions of sarcosine and DL-pipecolic acid the effect of sarcosine decreased as the concentration of DL-pipecolic acid increased (TABLE 4).

The chemical and physical properties of actinomycins II and III already have been described in detail (Johnson and Mauger, 1959). In connection with the hypothesis concerning competition for the "proline" site in the actinomycin peptides, I will mention only that when these actinomycins first were isolated by Goss and myself it was determined that actinomycin II contained only the amino acids valine, N-methylvaline, threonine, and sarcosine, whereas ac-

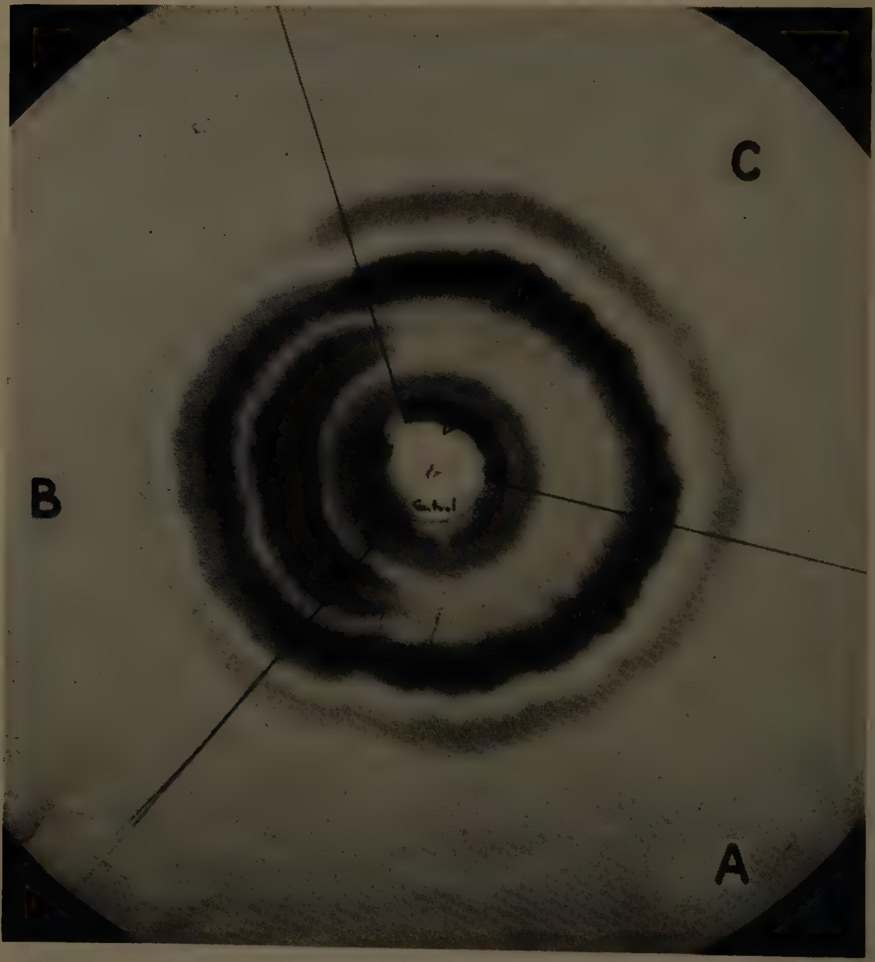

FIGURE 2. Circular paper chromatogram showing the actinomycin mixtures produced by *S. antibioticus*. Solvent system: 10 per cent aqueous solution of sodium *o*-cresotinate-di-*n*-butyl ether-*sym.*-tetrachloroethane (4:3:1 by volume). Sequence of actinomycins is from center to periphery; in all cases, the first zone beyond the origin constitutes biologically inactive, colored material. *A*, actinomycin mixture produced in the glutamic acid-galactose medium; actinomycins I, II (trace), III (trace), IV, and V. *B*, actinomycin mixture produced in the glutamic acid-galactose medium plus sarcosine, 250 µg./ml.; actinomycins I, II, II, IV, one unidentified component, and V. *C*, actinomycin mixture produced in the glutamic acid-galactose medium plus L-valine, 1000 µg./ml.; same as in *A*. Reproduced by permission of the *Biochemical Journal*.

tinomycin III contained these amino acids plus an amount of proline determined to be approximately one-half that found in actinomycin IV. Johnson has shown that actinomycin II possesses four moles of sarcosine and no proline, that actinomycin III has three moles of sarcosine and one mole of proline, and that actinomycin IV possesses two moles each of sarcosine and proline. We found also that the actinomycins synthesized in the presence of piperidine-2-carboxylic acid contained this imino acid. This has recently been confirmed by Johnson.

We have examined a number of analogues of piperidine-2-carboxylic acid to determine their effects upon actinomycin synthesis. These analogues include piperidine-2,6-dicarboxylic acid, 5-hydroxypiperidine-2-carboxylic acid, 4,5-dehydropiperidine-2-carboxylic acid, piperidine-3-carboxylic acid, and piperidine-4-carboxylic acids. They were found to be without effect, except piperidine-3-carboxylic acid, which proved to be a marked inhibitor of actinomycin formation (TABLE 5). The degree of inhibition depended in part on the concentration of the piperidine-3-carboxylic acid and in part on the length of incubation of the organism. Inhibition appears to be closely related to antibiotic formation, since the amount of mycelium produced in the presence of inhibitory levels of the compound was similar to that formed in its absence. Synthesis of actinomycin was blocked when the compound was supplied either before or during its course. Moreover, it was observed that L-proline at suitable concentrations reversed the inhibition due to piperidine-3-carboxylic acid. (Additional studies of the inhibition of actinomycin formation by this compound are in progress.) On the basis of the information obtained it seems that piperidine-3-carboxylic acid acts by blocking the incorporation of proline into the actinomycin peptides.

TABLE 3
PERCENTAGE OF COMPONENTS II AND III IN ACTINOMYCIN MIXTURES SYNTHESIZED IN THE PRESENCE OF SARCOSINE AND L-PROLINE*

Sarcosine (μg./ml.)	L-Proline (μg./ml.)	Actinomycins	
		II and III (%)	I, IV, and V (%)
Expt. 1			
100	0	62.4	37.6
100	500	59.2	40.8
100	1000	44.6	55.4
100	2000	18.2	81.8
Expt. 2			
500	0	57.0	43.0
500	500	55.5	44.4
500	1000	51.2	49.8
500	2000	47.1	53.0

* Sarcosine and proline were added to glutamic acid medium after 24-hour incubation. After 6 more days' cultivation the synthesized actinomycin mixture was harvested and the percentage of components was determined. Reproduced by permission from the *Biochemical Journal.*

Since the only differences among the actinomycin peptides produced by *S.*

antibioticus occur at the "proline" position, it is likely that under normal conditions of synthesis a competition exists between proline, hydroxyproline, 4-oxoproline and, to a slight extent, sarcosine for the "proline" site on some enzyme surface. If there exists a greater affinity for proline than for any of the

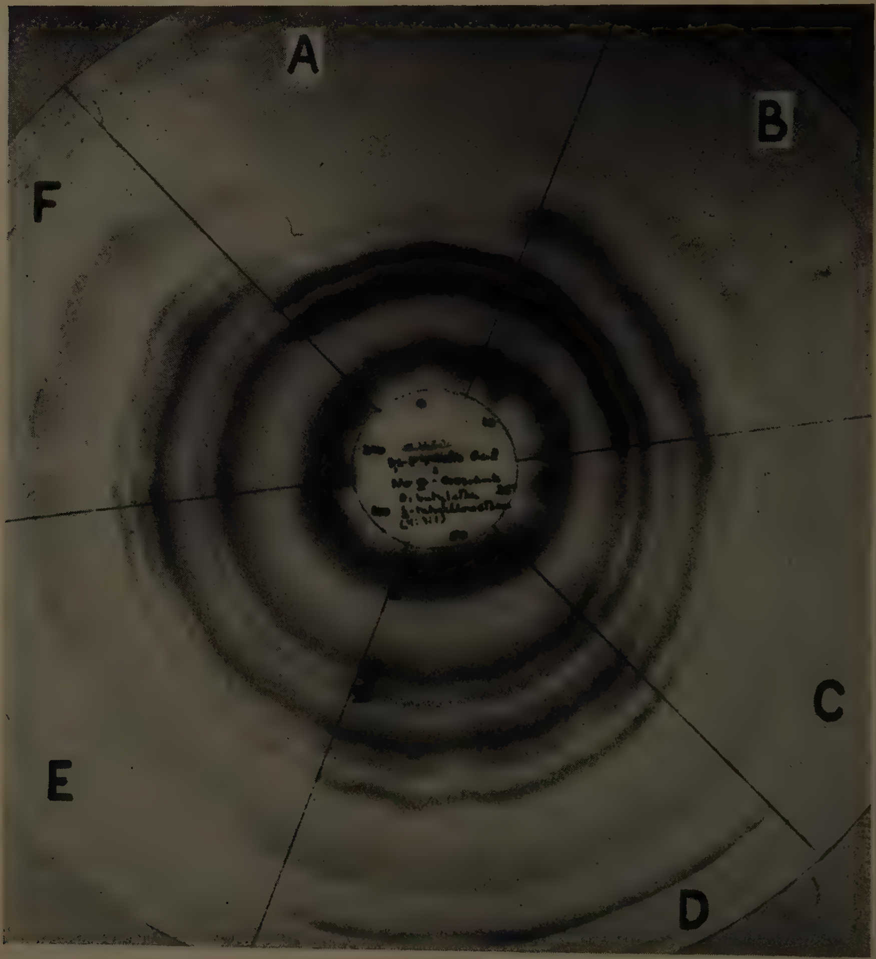

FIGURE 3. Circular paper chromatogram showing the actinomycin mixtures produced by *S. antibioticus*. Solvent system: 10 per cent aqueous solution of sodium *o*-cresotinate-di-*n*-butyl ether-*sym*.-tetrachloroethane (4:3:1 by volume). Sequence of actinomycins is from center to periphery; actinomycin I has not emerged from the biologically inactive, colored zone just beyond the origin. In all cases it appears that actinomycins I to V were formed. *A*, glutamic acid-galactose medium; actinomycins I to V and a sixth unidentified component. *B*, glutamic acid-galactose medium plus DL-pipecolic acid, 100 μg./ml.; actinomycins I to V and three additional components. *C*, glutamic acid-galactose medium plus DL-pipecolic acid, 250 μg./ml.; actinomycins I to V and four additional components. *D*, glutamic acid-galactose medium plus DL-pipecolic acid, 500 μg./ml.; actinomycins I to V and five additional components. *E*, glutamic acid-galactose medium plus DL-pipecolic acid, 1000 μg./ml.; actinomycins I to V and four additional components. *F*, glutamic acid-galactose medium plus DL-pipecolic acid, 2000 μg./ml.; actinomycins I to V and four additional components. Reproduced by permission of the *Biochemical Journal*.

other compounds mentioned, or if the concentration of proline at this site is greater than that of the other substances, one may expect this compound to be incorporated in the actinomycin peptides much more frequently than are the other amino acids. If the condensation of two molecules of 3-hydroxy-4-methylanthranilic acid peptide forms an actinomycin molecule (as is suggested by both Brockmann and Johnson), such should take place most frequently in the case of peptides that contain proline (actinomycin IV, 60 to 80 per cent;

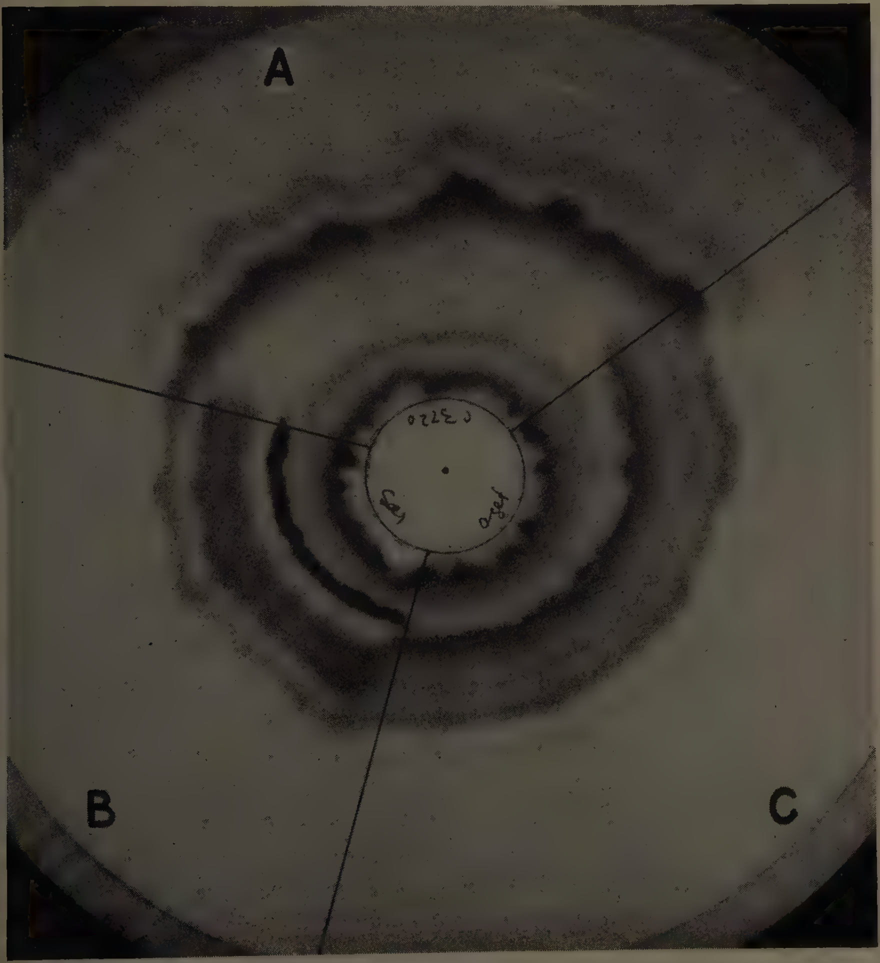

FIGURE 4. Circular paper chromatogram showing the actinomycin mixtures produced by *S. antibioticus*. Solvent system: 10 per cent aqueous solution of sodium *o*-cresotinate-di-*n*-butyl ether-*sym*.-tetrachloroethane (3:2:1 by volume). Sequence of actinomycins is from center to periphery; in all cases, the first zone beyond the origin constitutes biologically inactive, colored material. *A*, actinomycin mixture produced in the glutamic acid medium; actinomycins I, II and III (not visible in this mixture), IV, V. *B*, glutamic acid medium plus sarcosine, 250 μg./ml.; actinomycins I, II, IIIa, IIIb, IV, and V (trace). *C*, glutamic acid medium plus azetidine-2-carboxylic acid, 100 μg./ml.; actinomycin I, two to three new components, IV, and V.

Katz and Goss, 1958). The condensation of a peptide containing proline with one containing hydroxyproline (actinomycin I, 5 to 8 per cent), 4-oxoproline (actinomycin V, 5 to 25 per cent) or sarcosine (actinomycin III, 3 to 4 per cent) should occur much less frequently. Condensations involving two peptides, both containing hydroxyproline, 4-oxoproline, or sarcosine (actinomycin II, 2 to 3 per cent) probably also take place, but if so, very infrequently, and components so produced would be extremely difficult to detect by the chromatographic methods available.

TABLE 4
INFLUENCE OF SARCOSINE AND DL-PIPECOLIC ACID ON THE SYNTHESIS OF ACTINOMYCINS II AND III*

DL-Pipecolic acid (μg./ml.)	Sarcosine (μg./ml.)	Actinomycin components (%)	
		Actinomycins II and III	Pipecolic acid components
0	0	6.8	0
0	250	67.2	0
5	250	62.7	5.4
10	250	57.1	14.4
25	250	38.8	18.9
100	250	16.2	44.5
100	0	6.1	48.2

* Sarcosine and DL-pipecolic acid were added to glutamic acid medium after 36-hour incubation. On day 7 of cultivation the synthesized actinomycin mixture was harvested and the percentage of components was determined. Reproduced by permission from the *Biochemical Journal.*

TABLE 5
INHIBITION OF ACTINOMYCIN SYNTHESIS (*S. ANTIBIOTICUS*) BY PIPERIDINE-3-CARBOXYLIC ACID*

Piperidine-3-carboxylic acid (μg./ml.)	Actinomycin titer after 6 days' incubation (μg./ml.)	Inhibit. (%)
0	63	0
10	59	6
50	32	49
250	17	73
500	4	94

* Piperidine-3-carboxylic acid was added to the glutamic acid medium 24 hours after inoculation of *S. antibioticus.*

If one modifies the nutritive environment—for example, by changing the nitrogen source (Katz *et al.*, 1958), by adding a second one (Schmidt-Kastner, 1956*a*,*b*; Katz and Goss, 1958), or by changing the carbon-nitrogen ratio (Martin and Pampus, 1956)—different equilibriums may arise between the competing amino acids of a given system. In this newly established condition a different actinomycin component may become the major one of a mixture or, at least, increase more than the others.

The introduction of an exogenous supply of one of the "variable" amino acids in a competing system may shift the equilibrium considerably, as was the case of hydroxyproline and actinomycin I (Katz and Goss, 1958), sarcosine and actinomycins II and III with *S. antibioticus* (Katz and Goss, 1958, 1959), and DL-valine and actinomycin IV with *S. chrysomallus* (Schmidt-Kastner, 1956*b*).

Adding a substance closely related to an amino acid normally found in actinomycin may cause a competition between the endogenously synthesized substance and the exogenous compound; incorporation of such related substances into the peptides might occur with the formation of new actinomycins. Piperidine-2-carboxylic acid and azetidine-2-carboxylic acid appear to be incorporated directly into actinomycin molecules in this manner.

An exogenous compound may first be altered by the organism; the modified compound then competes with a naturally synthesized amino acid for the latter's site in the actinomycin peptide. For example, *S. chrysomallus* converts added isoleucine to *N*-methylisoleucine, and the methylated amino acid competes with endogenously formed *N*-methylvaline. One or two molecules of the former amino acid may be introduced into the "*N*-methylvaline" position of the actinomycin molecule; as a result, two new actinomycins are synthesized by the organism (Schmidt-Kastner, 1956*a*,*b*).

The number of new actinomycins synthesized in any given situation will depend on the amino acid, the concentration used, and the organism. *A priori*, one would expect several new compounds to be produced in each instance in which incorporation takes place. For example, with piperidine-2-carboxylic acid (pipecolic acid) and *S. antibioticus* it should be possible to find actinomycin peptides containing (1) pipecolic acid/pipecolic acid, (2) proline/pipecolic acid, (3) hydroxyproline/pipecolic acid, and (4) oxoproline/pipecolic acid. Moreover, the isomer of each actinomycin containing asymmetrical peptides probably is synthesized. With sarcosine and *S. antibioticus* one should find sarcosine/sarcosine and sarcosine/proline peptides (Johnson and Mauger, 1959) and, in addition, sarcosine/hydroxyproline and sarcosine/ketoproline peptides. Isomers of the last three combinations also should be formed.

Biogenesis of D-Amino Acids

Thorne *et al.* (1955) and Thorne and Molnar (1955) have demonstrated that D-glutamic acid and certain other D-amino acids were formed by transaminase-catalyzed reactions when cell-free extracts of *Bacillus subtilis* and *B. anthracis* were employed. These organisms synthesize considerable amounts of a D-glutamyl polypeptide when grown in a medium containing L-glutamic acid. D-Glutamic acid does not serve as a suitable substitute for the L-isomer. Moreover, tracer studies with C^{14}-labeled L- and D-glutamic acid have revealed that the L-isomer is preferentially utilized for synthesis of the D-glutamyl peptide. A glutamic acid racemase in these organisms has not been demonstrated. However, the discovery that D- as well as L-amino acid transamination reactions occur and that an alanine racemase is present in extracts of these organisms suggested the following series of reactions as a mechanism of indirect conversion of L-glutamic acid to the D-isomer: (1) transamination between pyruvic acid and L-glutamic acid, producing L-alanine and α-ketoglutaric acid, (2) alanine racemization, and (3) transamination between α-ketoglutaric acid and D-alanine to form D-glutamic acid.

Thus far we have been unable to find a similar system operating in *S. antibioticus* or *S. chrysomallus* that would explain how D-valine and D-alloisoleucine are formed. Cell-free extracts of these organisms do mediate transamination reactions involving L-amino acids. In addition, we have obtained some preliminary evidence that an alanine racemase is present in cell-free extracts. However, it has not been possible to show the existence of a transaminase system for the synthesis of D-amino acids. Conceivably, a racemase is responsible for D-valine and D-alloisoleucine formation but tne possibility has not yet been explored.

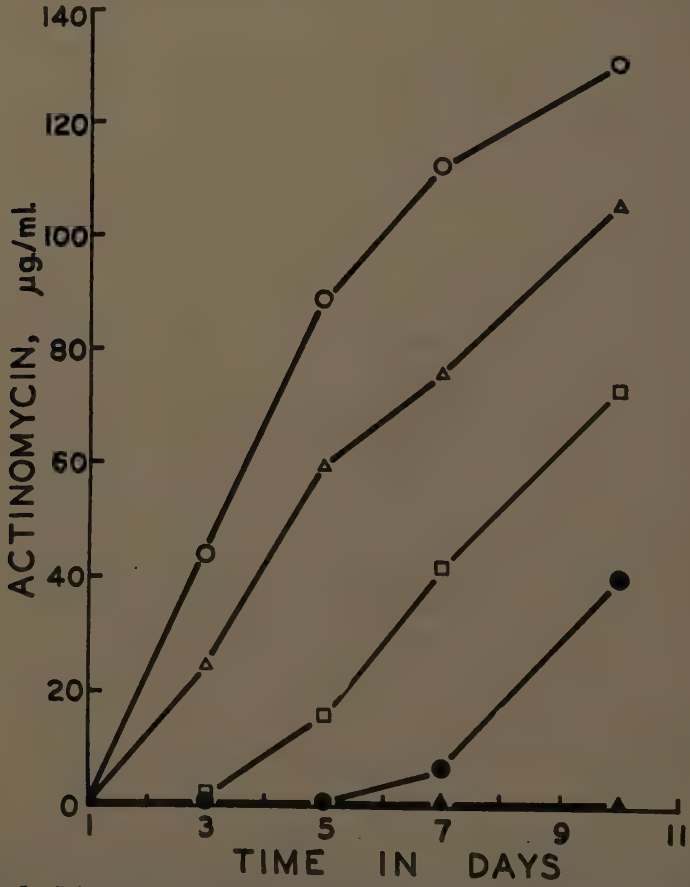

FIGURE 5. Inhibition of actinomycin synthesis by D-valine. *S. antibioticus* was grown in the glutamic acid medium to which D-valine was added 24 hours after inoculation: ○, control (no D-valine); △, 10 μg./ml.; □, 50 μg./ml.; ●, 100 μg./ml.; ▲, 200 μg./ml. Reproduced by permission of the *Journal of Biological Chemistry*.

During an investigation concerned with the biogenesis of D-amino acids by these organisms it was observed that the addition of D-valine, D-isoleucine, or D-alloisoleucine to a chemically defined medium in which *S. antibioticus* was growing resulted in a marked inhibition of actinomycin formation (Katz, 1959 and 1960). The parallel between these results and those of studies in penicillin biosynthesis with D-valine and DL-isoleucine (Demain, 1956; Arnstein and Margreiter, 1958) prompted a further investigation of the phenomenon.

The degree to which actinomycin synthesis is inhibited depends on the concentration of D-valine used and the length of incubation of the organism (FIGURE 5). From experiment to experiment, some variation in the concentration of D-isomer necessary to achieve a given level of inhibition was observed, but the effect was consistently obtained. DL-Valine also has a striking effect upon actinomycin formation, the reduction in antibiotic titer being due solely to its D-valine content (TABLE 6). Inhibition by D-valine appears to be related directly to antibiotic synthesis, since the amount of mycelium synthesized in the presence of inhibitory concentrations of the D- isomer was found to be similar to that formed with L-valine or when no valine was added.

TABLE 6

INFLUENCE OF VALINE ON ACTINOMYCIN SYNTHESIS AND GROWTH OF *S. ANTIBIOTICUS*

Valine isomer	Concent. (μg./ml.)	Actinomycin titer after 5 days (μg./ml.)	Inhibit. or stimul. (%)	Mycelium dry wt. after 5 days* (mg./100 ml.)
None	0	89	0	147.4
L-	10	89	0	144.0
D-	10	60	−33	147.0
DL-	20	65	−27	—
L-	50	86	−3	151.5
D-	50	16	−82	155.6
DL-	100	14	−84	—
L-	100	93	+5	155.0
D-	100	0	−100	157.5
DL-	200	0	−100	—

* These data are taken from a second experiment in which the actinomycin titer in the different test series was found to be higher. The per cent inhibition observed in the presence of D-valine was similar to that reported above, however. In each case as shown in TABLE 6 inhibition was similar. Reproduced by permission from the *Journal of Biological Chemistry.*

D-Valine proved an effective inhibitor when supplied prior to or during the course of actinomycin synthesis (FIGURE 6). For example, the D-isomer (250 μg./ml.) added to cultures of *S. antibioticus* after three, five, or seven days' incubation checked further synthesis. The antibiotic titer of the culture medium actually decreased during incubation, presumably because of destruction of the antibiotic previously formed. The D-valine, supplied before actinomycin synthesis occurred, completely inhibited production for about seven days; however, the inhibition was overcome to some extent during the late stages of cultivation.

It was observed that L-valine, over a wide concentration range, was not inhibitory. In some experiments it appeared to stimulate actinomycin synthesis, suggesting that formation of this amino acid may be rate-limiting for actinomycin production. It was also found that the L-compound, at suitable concentrations, reversed the inhibition due to D-valine (TABLE 7).

Some other compounds chemically related to D-valine also were quite inhibitory. For example, α-methyl-DL-valine, a valine analogue that inhibits penicillin biosynthesis (Demain, 1956), also prevents actinomycin formation (TABLE 7). The inhibition was not as striking as that seen with D-valine, however. It is interesting to note that L-valine also reversed the inhibition due to α-methyl-DL-valine. Finally, D-alloisoleucine and D-isoleucine, but not L-isoleucine, were found to be appreciably inhibitory (TABLE 8).

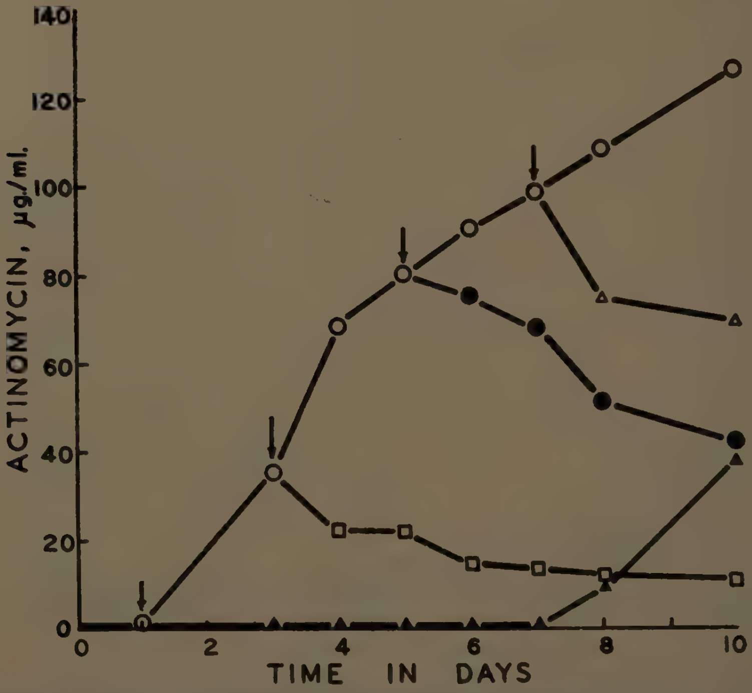

FIGURE 6. Influence of addition of D-valine during actinomycin synthesis. *S. antibioticus* was grown in the glutamic acid medium; D-valine, 250 μg./ml., was added as indicated by arrows: ○, control (no D-valine); ▲, 1 day; □, 3 days; ●, 5 days; △, 7 days. Reproduced by permission of the *Journal of Biological Chemistry.*

The observation that L-valine reverses the effect of D-valine and α-methyl-DL-valine and may even stimulate actinomycin formation when used alone suggests that L-valine in preference to D-valine is used for synthesis of the D-valine present in an actinomycin molecule. Through studies with isotopically labeled D- and L-valine it should be possible to provide a more conclusive answer to this question. The penicillamine moiety of penicillin (derived from valine) has the D- configuration; moreover, one finds D-valine residues in valinomycin. In both cases isotopic studies with C^{14}-labeled D- and L-valine have shown that L-valine is preferentially utilized for synthesis of the D-valine moiety (Arnstein and Margreiter, 1958; MacDonald, 1960). On the basis of these findings, I should not be surprised to find that L-valine is the actual precursor of the D-valine present in an actinomycin molecule.

The biogenesis of D-valine and D-alloisoleucine and the site and nature of the

TABLE 7

INHIBITION OF ACTINOMYCIN SYNTHESIS BY D-VALINE AND α-METHYL-DL-VALINE AND ITS REVERSAL BY L-VALINE

Expt. no.	Inhibitory amino acid	Concent. (μg./ml.)	L-Valine (μg./ml.)	Inhibit. actinomycin production	
				After 3 days (%)	After 7 days (%)
1	None	0	0	0*	—
	D-Valine	10	0	65	—
		10	1000	0	—
		50	0	100	—
		50	500	76	—
		50	1000	12	—
		250	0	100	—
		250	500	88	—
		250	1000	41	—
2	None	0	0	—	0*
	α-Methyl-DL-valine	500	0	—	17
		500	250	—	7
		500	500	—	0
3	None	0	0	—	0*
	α-Methyl-DL-valine	1000	0	—	24
		1000	500	—	7
		1000	1000	—	2

* Actinomycin titer: after 3 days (expt. 1) 29 μg./ml.; after 7 days (expt. 2) 71 μg./ml.; and (expt. 3) 86 μg./ml. Reproduced by permission of the *Journal of Biological Chemistry.*

TABLE 8

INHIBITION OF ACTINOMYCIN SYNTHESIS BY D-ISOLEUCINE AND D-ALLOISOLEUCINE

Inhibitory amino acid	Concent. (μg./ml.)	Inhibit. actinomycin production	
		After 4 days (%)	After 10 days (%)
None	0	0*	0*
D-Isoleucine	25	46	3
	50	63	13
	100	91	33
	250	94	84
D-Alloisoleucine	25	51	17
	50	86	39
	250	100	77
	500	100	94

* Actinomycin titer: after 4 days, 35 μg./ml.; after 10 days, 90 μg./ml. Reproduced by permission of the *Journal of Biological Chemistry.*

inhibition of actinomycin synthesis in the presence of these amino acids are still to be investigated.

Actinomycin Chromophore Biosynthesis

Biochemical investigations with *Drosophila* mutants requiring kynurenine or 3-hydroxykynurenine (Butenandt, 1949), and radioisotope experiments with C^{14}-labeled tryptophan and kynurenine in *Vanessa urticae* and *Calliphora erythrocephala* (Butenandt and Neubert, 1955; Butenandt and Beckmann, 1955), have established that tryptophan is the precursor of certain insect pigments, the ommochromes, which possess a phenoxazinone nucleus similar to that found in actinomycin. The immediate precursor of these pigments probably is 3-hydroxykynurenine derived from the oxidative metabolism of tryptophan.

In a purely chemical system, derivatives of the actinomycin chromophore can be synthesized readily through an oxidative condensation of certain orthoaminophenols. For example, the condensation of 3-hydroxy-4-methylanthranilic acid with 2,5-dihydroxytoluquinone gives rise to actinocinin (Brockmann and Muxfeldt, 1956*a*); in addition, the self-condensation of 3-hydroxy-4-methylanthraniloylglycine methyl ester yields actinocyl-bis-glycine methyl ester (Brockmann and Muxfeldt, 1956*b*). It has been postulated, therefore, that 3-hydroxy-4-methylanthranilic acid, *in vivo*, is the immediate precursor of the phenoxazinone chromophore (Johnson, 1956). Presumably, an actinomycin-producing organism metabolizes tryptophan via kynurenine and 3-hydroxykynurenine to 3-hydroxyanthranilic acid. The latter compound then might undergo methylation at the 4-position to yield the 3-hydroxy-4-methylanthranilic acid. *S. antibioticus* does metabolize tryptophan to 3-hydroxyanthranilic acid via kynurenine and 3-hydroxykynurenine (Sivak and Katz, 1959). A study of the methylation of 3-hydroxyanthranilic acid has not been attempted.

Fermentation studies with *S. antibioticus* and unlabeled tryptophan or certain of its metabolites—for example, kynurenine, 3-hydroxykynurenine, or 3-hydroxyanthranilic acid—failed to demonstrate that any of these compounds is important in actinomycin chromophore synthesis. Evidence for the role of tryptophan in chromophore biogenesis was obtained with 7-α-C^{14}-labeled DL-tryptophan. The isotope was supplied to growing cells of *S. antibioticus* and, subsequently, the actinomycin produced was isolated, diluted with cold actinomycin IV, and crystallized to constant radioactivity. Actinocinin and the dimethylester of desaminoactinocylthreonine (chromophore derivatives obtained from actinomycin by acid degradation) were found to possess essentially the same specific activity as the intact actinomycin molecule (TABLE 9). The amino acids of the actinomycin peptides were not labeled. While these results do not prove conclusively that tryptophan is the precursor of the chromophore, the evidence does suggest that the benzene ring of tryptophan was incorporated intact into the actinomycin chromophore.

Birch (1958) proposed that acetate was the precursor of the 3-hydroxy-4-methylanthranilic acid unit. It was advanced that four acetic acid molecules might give rise to the anthranilic acid precursor through a process of oxidation, reduction, and amination. We have carried out an experiment with $C^{14}H_3$-labeled acetate to determine whether acetate is important for chromophore biogenesis. Our data suggest that acetate is not a precursor of the chromophore (TABLE 10). The actinomycin produced in the presence of the labeled acetate had a specific activity of 3.6×10^5 cpm/mmole; the actinocinin chromophore possessed a specific activity of 1.45×10^4 cpm/mmole, or about 4 per cent of the total activity of the actinomycin. On the other hand, the amino acids, particularly proline, contained considerable radioactivity.

Additional evidence to support the view that tryptophan is the precursor of the actinomycin chromophore was obtained with the tryptophan analogues, α-, 4-, 5-, and 6-methyltryptophan. My associates and I observed that these compounds were quite inhibitory to actinomycin synthesis with growing cells and washed suspensions of *S. antibioticus* (FIGURE 7); the order of their effectiveness was α- > 4- > 5- > 6-methyltryptophan. It was found that tryptophan and kynurenine reversed the inhibition due to α- and 4-methyltryptophan (TABLE 11).

TABLE 9
INCORPORATION OF 7-α-C^{14}-DL-TRYPTOPHAN INTO THE ACTINOMYCIN CHROMOPHORE BY *S. ANTIBIOTICUS**

Product	Specific activity (cpm/mmole)
Actinomycin	1.46×10^6
Actinocinin	1.52×10^6
Desaminoactinocylthreonine	1.42×10^6

* Amino acids were not labeled.

TABLE 10
INCORPORATION OF $C^{14}H_3$-LABELED COONA INTO THE ACTINOMYCIN CHROMOPHORE BY *S. ANTIBIOTICUS**

Preparation	Specific activity (cpm/mmole)	Activity (%)
Actinomycin	3.6×10^5	—
Actinocinin	1.5×10^4	4

* Amino acids were labeled.

Biochemical Origin of the Methyl Groups Present in the Chromophore, Sarcosine, and N-Methylvaline

Our investigations in this connection are rather fragmentary, but the evidence suggests that methionine may participate in the methylation of the actinomycin chromophore and the amino acids sarcosine and *N*-methylvaline. When $C^{14}H_3$-labeled L-methionine had been supplied to *S. antibioticus*, the actinomycin subsequently isolated had a specific activity of 3.0×10^6 cpm/mmole. Only sarcosine and *N*-methylvaline of the amino acids present in actinomycin possessed radioactivity. The actinocinin, obtained by acid hydrolysis, was found to contain approximately one third the total activity of the actinomycin. Further detailed investigations of the role of methionine in actinomycin synthesis are in progress.

Summary

Increased synthesis of actinomycin components normally formed in trace amounts occurred when hydroxyproline or sarcosine was supplied to *Streptomyces antibioticus*. In addition, the synthesis of new actinomycins took place when the proline analogue piperidine-2-carboxylic acid or azetidine-2-carboxylic

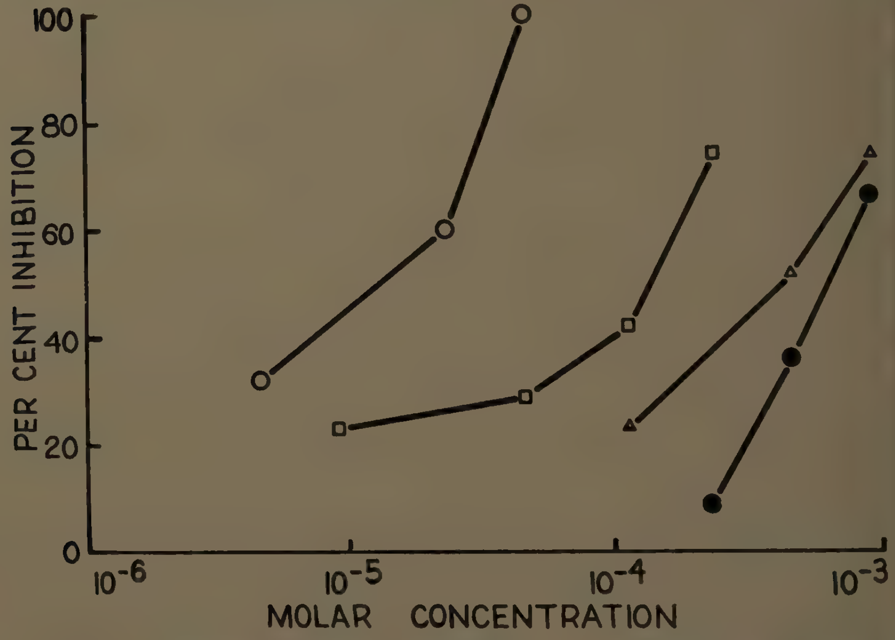

FIGURE 7. Inhibition of actinomycin synthesis by α-, 4-, 5-, and 6-methyl-DL-tryptophan. *S. antibioticus* was grown in the glutamic acid medium and the methylated analogues of tryptophan were added at the time actinomycin production began. ○, α-; □, 4-; △, 5-; ●, 6-.

TABLE 11

REVERSAL OF α-METHYL-DL-TRYPTOPHAN INHIBITION OF ACTINOMYCIN SYNTHESIS BY L-TRYPTOPHAN AND L-KYNURENINE*

α-Methyl-DL-tryptophan (μg./ml.)	L-Tryptophan (μg./ml.)	L-Kynurenine (μg./ml.)	Reversal of Inhibition (%)	
			9 hr.	23 hr.
2	0	0	0	0
2	10	0	76	53
2	20	0	100	73
2	40	0	100	89
2	0	10	12	29
2	0	20	80	53
2	0	40	100	76

* Washed suspensions of *S. antibioticus* in .067 *M* phosphate buffer, *p*H 7.0.

acid was added to the medium. D-Valine, D-alloisoleucine, D-isoleucine, and α-methyl-DL-valine proved inhibitory to actinomycin formation. L-Valine reversed the inhibition due to D-valine and α-methyl-DL-valine and, when used alone, stimulated actinomycin synthesis. The data obtained suggest that L-valine is the precursor of the D-valine in the actinomycin peptides. Results of inhibitor studies with α-, 4-, 5-, and 6-methyltryptophan and the incorporation of the radioactivity of 7-α-C^{14}-labeled DL-tryptophan into the actinomycin chromophore favor the view that tryptophan plays an important role in the biosynthesis of the phenoxazinone chromophore of actinomycin. Preliminary studies with $C^{14}H_3$-labeled methionine indicate that this amino acid may contribute its methyl group to the actinomycin chromophore, to sarcosine, and to *N*-methylvaline.

Acknowledgments

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STRUCTURAL DIFFERENCES OF THE ACTINOMYCINS AND THEIR DERIVATIVES

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About nine years ago, in 1951, it was observed in the laboratory of my associates and myself that the well-crystallized actinomycin C, isolated from cultures of *Streptomyces chrysomallus*,[1] was a mixture of three different actinomycins that could be separated by fractionated countercurrent distribution.[2] Not long afterward we found solvent systems in which the three actinomycins (named C_1, C_2, and C_3) could also be separated by means of paper chromatography or partition chromatography on cellulose.[3] In the application of these methods to the analysis of other actinomycins—for instance, to the actinomycin B of Todd and his co-workers,[4] the actinomycin X[5] isolated in our laboratory or, later, the actinomycin A of Waksman and Tishler[6]—it was shown that they also were mixtures of different actinomycins. This result has led to the conclusion that all *Streptomyces* strains able to synthesize actinomycins will usually produce several actinomycins side by side.

When methods of separating an actinomycin mixture were at our disposal, we set up the following program for further research:

(1) Discovery of as many actinomycin-producing strains as possible; we wanted to know how many actinomycins might be synthesized by microorganisms.

(2) Analysis of the structure of one of the actinomycins.

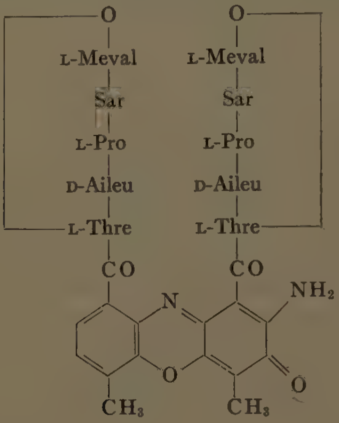
(3) Differentiation of the structures of the various actinomycins.

(4) Preparation of actinomycin derivatives in order to find out whether there are derivatives having better cytostatic activities than the actinomycins themselves.

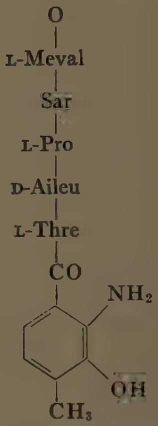
Here will be given a brief account of our results concerning (1) the structural differences of a number of actinomycins and (2) some derivatives of the actinomycins. In order to determine first, how many different actinomycins can be synthesized by microorganisms, we analyzed with our separation methods the actinomycin mixtures of 21 strains selected from about 2000 *Streptomyces* strains. We found[3] that all these mixtures consisted of one or two main components and several actinomycins that were present only in small quantities. The chief components of the different mixtures were actinomycins C_1 or C_2 and C_3, or actinomycin X_2 and $X_{0\beta}$.[5,7,8] These five principal actinomycins we investigated first.

After the researches of Todd, Johnson and his co-workers, and of the workers in our laboratory had shown that the actinomycins are chromopeptides, our first task was to find out whether the five principal actinomycins differ only in the structure of their peptide moiety or also in the structure of their chromophoric moiety. By degradation experiments we were able to show that they all contain the same chromophoric moiety[9] and therefore differ only in the structure of their peptide moiety.

The next problem to be solved was that of elucidating the structure of only one of the actinomycins. For this investigation we used actinomycin



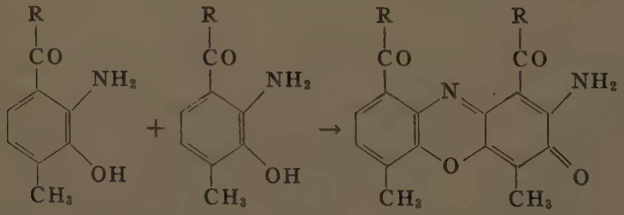
I



II

C_3 and, in 1956, were able to propose the structure given by Formula I.[10]

The chromophoric part of the molecule, responsible for the yellow-red color of the actinomycins, is the 2-amino-4:6-dimethyl-3-oxo-phenooxazine-1:9-dicarboxylic acid, which we have named actinocin (Formula IV). Actinocin is bonded with its two carboxy groups to two peptide-lactone groups of equal structure. The two *N*-methylvaline residues at the end of each peptide chain are esterified with the hydroxy group of the two threonine residues. Until the beginning of this year it was not known whether the carboxy groups of the two *N*-methylvaline residues were esterified with the threonine residue of the same peptide chain as that in Formula I or were bonded to the threonine of the other peptide chain. But recently P. Boldt in our laboratory was able to ascertain that the two lactone groups have the position suggested by Formula I.

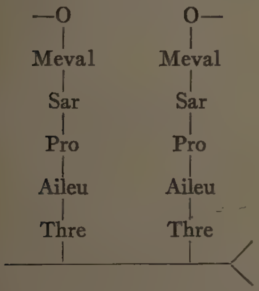


III R = OH
IIIa R = peptide
III R = OH
IIIa R = peptide
IV R = OH
IVa R = peptide

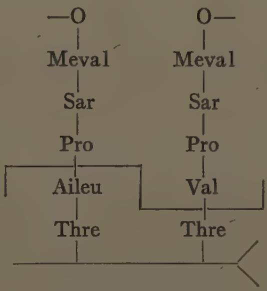
As we have found,[10] actinocin (Formula IV, the chromophoric moiety of actinomycin C_3 and of the other principal actinomycins) can be prepared in an almost quantitative yield by oxidative condensation of two molecules of 3-hydroxy-4-methylanthranilic acid (Formula III). Furthermore, starting with compounds of the constitution of III*a*, we could synthesize a number of chromopeptides (IV*a*) containing actinocin, which is bonded with its carboxyl groups to the amino groups of dipeptides, tripeptides, and tetrapeptides. The observation that such condensations may be carried out at room temperature and *p*H 7.8, that is, under biological conditions, led us to the assumption that *in vivo* the actinomycins are synthesized in the same way: in the case of actinomycin C_3, for instance, by oxidative condensation of two molecules of the precursor II.

If this assumption is correct, the combination of only a few different precursors would give a great number of different actinomycins: a simple calculation shows, for instance, that from six different precursors a cell could synthesize 36 different actinomycins, provided that all facilities for the combination of two molecules of the precursors are realized.

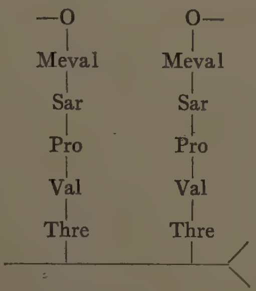
For the following discussion of the structural differences of the actinomycins it will be practicable to simplify their structural formulas in a manner that, in the case of actinomycin C_3, is demonstrated by Formula V. The ring system of the chromophoric moiety (actinocin) is symbolized by a horizontal line and its amino and carbonyl groups by two short diagonal dashes. The Roman characters indicating configuration of the amino acids are omitted, and the bond between the *N*-methylvaline and the threonine is represented by a short dash.



V Actinomycin C_3



VI Actinomycin C_2



VII Actinomycin C_1(D)

Actinomycin C_1, one of the components of the actinomycin C mixture, differs from actinomycin C_3 only in containing two molecules of D-valine in place of two molecules of D-alloisoleucine. Therefore it was natural to assume, on the basis of our actinomycin C_3 formula (I), that actinomycin C_1 has the constitution given in Formula VII. We were able to prove [12] that this is the case, in the same manner as with actinomycin C_3.[13] In this connection it must be mentioned that on the basis of degradation experiments, Bullock and Johnson[14] came to the conclusion that actinomycin D, described for the first time in 1954 by Vining and Waksman,[15] also has the constitution given by Formula VII, thus proving unequivocally that actinomycin D is identical with our actinomycin C_1, described for the first time in 1952.[2]

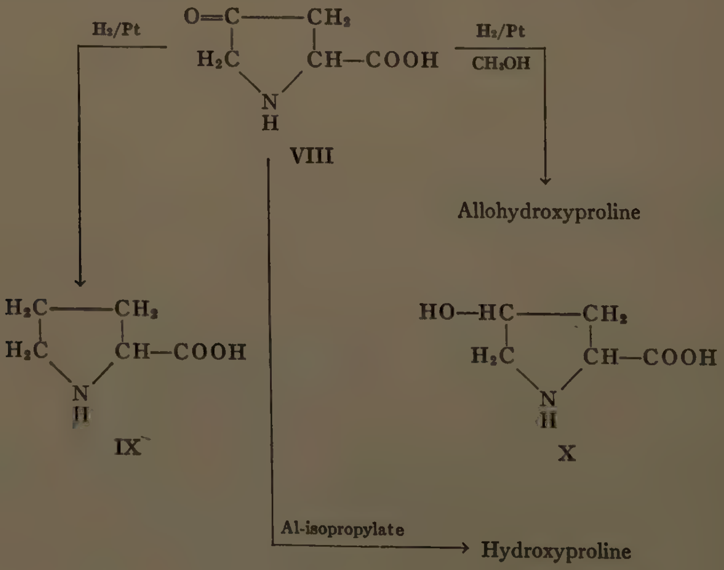
In contrast to actinomycin C_1, which contains *two* molecules of valine, and to actinomycin C_3, which contains *two* molecules of D-alloisoleucine, actinomycin C_2 has *one* molecule of D-valine and *one* molecule of D-alloisoleucine, thus holding a central position between actinomycins C_1 and C_3. On the basis of degradation experiments we have shown[12] that actinomycin C_2 may be given the Formula VI in which, however, the positions of the valine and alloisoleucine are not yet demonstrated in all details: it is possible that valine and alloisoleucine are to be exchanged, as indicated by the serpentine line.

At first it could not be disproved that actinomycin C_2 is a mixture of two actinomycins of the Formula VI, one of which has the valine in the right peptide group and the other in the left peptide group. However, recently we have shown by hydrolytic degradation that our actinomycin C_2 is homogeneous and not a mixture of two isomers. According to our experiments, C_2 seems to have the valine in the right peptide group in Formula VI, but we are not quite sure.

In connection with Formula VI, it is of interest that some time ago we isolated from the actinomycin C complex a small yield of crystallized actinomycin, which is an isomer of actinomycin C_2 that we have termed actinomycin C_{2a}.[16] Probably this is the isomer of actinomycin C_2 the existence of which is predicted by our hypothesis concerning the biosynthesis of the actinomycins. This means that, if actinomycin C_2 is constituted as shown in Formula VI (valine in the right peptide group), then actinomycin C_{2a} would have the constitution indicated in Formula VI, but with the valine and alloisoleucine interchanged according to the serpentine line.

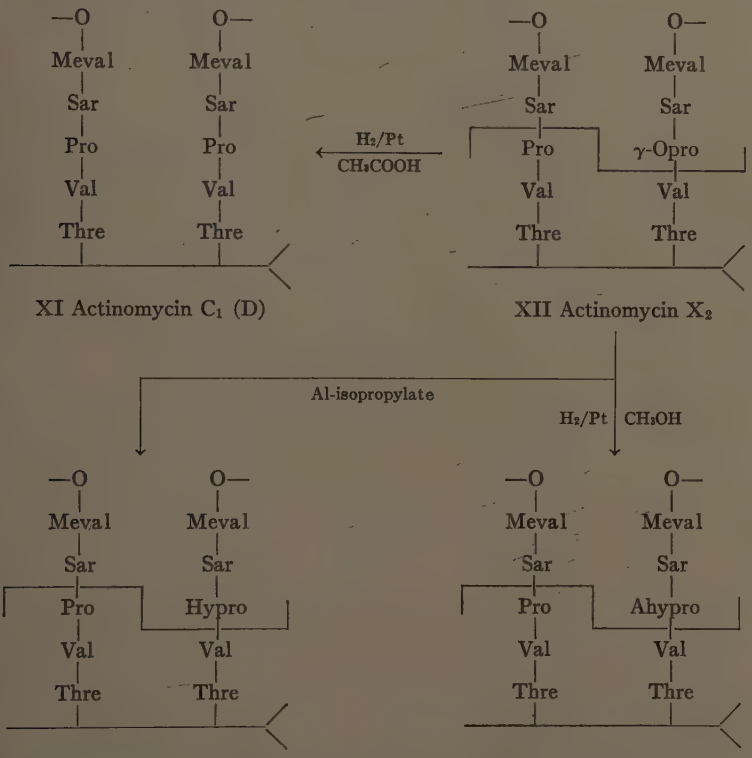
Of the five principal actinomycins, $X_{0\beta}$ and X_2 still remain to be discussed. Actinomycin $X_{0\beta}$ differs from C_1 (Formula VII) in that instead of one molecule of proline (IX) it contains one molecule of hydroxyproline (X). Actinomycin X_2 differs from actinomycin C_1 (VII) in containing one molecule of γ-oxoproline (VIII) instead of one molecule of proline.

By hydrogenation with platinum catalyst in acetic acid, the γ-oxoproline (in the form of carbethoxy-oxoproline-ethylester) is reduced to proline (Formula IX), whereas hydrogenation with the same catalyst in methanol gives the allohydroxyproline (X).[17] If, on the other hand, the reduction of γ-oxo-



proline (in the form of carbethoxy-oxoproline-ethylester) is carried out with aluminum-isopropylate, a mixture of hydroxyproline and allohydroxyproline is produced.[17]

The same reductions are possible when the γ-oxoproline (Formula VIII) is part of the peptide group of actinomycin X_2. Thus by hydrogenation with platinum catalyst in acetic acid, actinomycin X_2, containing one mole of proline and one molecule of oxoproline (VIII) is converted to an actinomycin containing two molecules of proline. This actinomycin was found to be identical with actinomycin C_1 (XI), a finding that shows that the amino and imino acids of actinomycins C_1 and X_2 have the same sequence. For this reason Formula XII may be given to actinomycin X_2. It remains to be determined which peptide group of this formula contains the proline and which the γ-oxoproline.



XI Actinomycin C_1 (D)

XII Actinomycin X_2

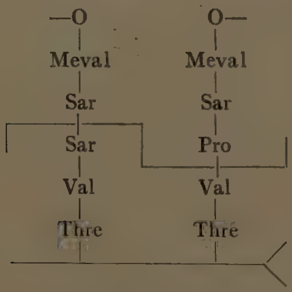
XIII Actinomycin $X_{0\beta}$

XIV Actinomycin $X_{0\delta}$

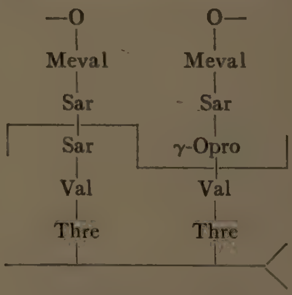
On reduction with aluminum-isopropylate, X_2 is converted to an actinomycin that contains one molecule of hydroxyproline (Formula X) and one mole of proline (IX); in other words, the γ-oxoproline (VIII) of X_2 is reduced to hydroxyproline (X). The crystallized reduction product was shown to be identical with actinomycin $X_{0\beta}$. Therefore, on the basis of Formula XII of actinomycin X_2, Formula XIII could be given to actinomycin $X_{0\beta}$ and, as in the case of actinomycin X_2, there remains to be established the position of the hydroxyproline, which may be located either in the left peptide group or in the right, as indicated by the serpentine line in Formula XIII.

On reduction with platinum catalyst in methanol the γ-oxoproline (Formula VIII) of actinomycin X_2 is reduced to allohydroxyproline (X), and an actinomycin is formed that we have termed actinomycin $X_{0\delta}$. It is a stereoisomer of actinomycin $X_{0\beta}$, differing from actinomycin $X_{0\beta}$ only in the configuration of that carbon atom of hydroxyproline which is bonded to the hydroxyl group. This small difference is sufficient to cause a marked difference in the solubility of the two actinomycins and also of their antibiotic activity. In methanol containing 5 per cent water, actinomycin $X_{0\delta}$ is three times as soluble as actinomycin $X_{0\beta}$ and against *Bacillus subtilis* it is at least five times as active as actinomycin $X_{0\beta}$.

Instead of proline, hydroxyproline, or γ-oxoproline, the cell can incorporate sarcosine into the peptide chain. From a *Streptomyces* strain producing the actinomycin X complex we were able to isolate in a small yield two actinomycins that we have named actinomycin X_{1a} and $X_{0\gamma}$. Actinomycin X_{1a} contains three molecules of sarcosine and one molecule of proline, and X_{1a} also has three molecules of sarcosine, but it has one molecule of γ-oxoproline (Formula VIII). The sequences of the amino acids of these two actinomycins have not yet been determined. However, they probably are the same as given in Formulas XV and XVI; perhaps sarcosine and proline (XV) or sarcosine and oxoproline (XVI) should be interchanged according to the serpentine line.



XV Actinomycin $X_{0\gamma}$

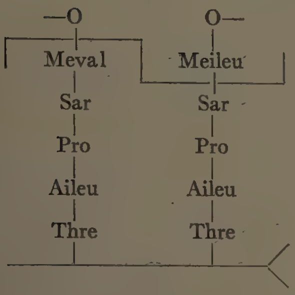


XVI Actinomycin X_{1a}

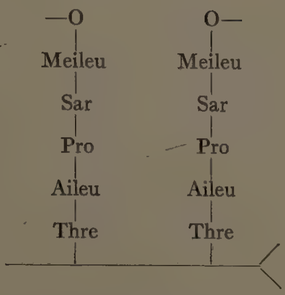
Actinomycin $X_{0\gamma}$ contains the same amino and imino acids as does another actinomycin, recently isolated by Katz and Goss[18] and analyzed by Johnson and Mauger,[19] that has been named actinomycin III. Whether they are identical or isomeric is still uncertain.

Only a few remarks will be made on those actinomycins, discovered by Schmidt-Kastner,[20] that are produced by "directed biosynthesis." In cooperation with Schmidt-Kastner we have made quantitative determinations of the amino acids of the actinomycins E and F of Schmidt-Kastner. We have also pointed out those amino acids of the peptide part which are esterified with the threonine residues.

We cannot yet give an exact proof of any sequence of the amino acids of the E and F actinomycins. However, if it is assumed that the directed biosynthesis is controlled by a tendency to make the structural differences



XVII Actinomycin E_1

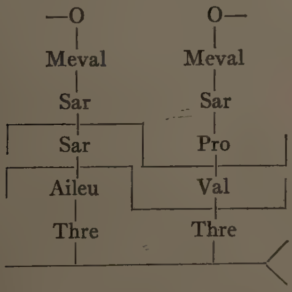


XVIII Actinomycin E_2

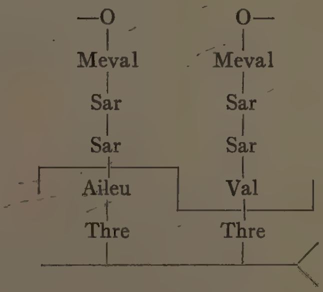
between the E and F and the normal actinomycins* as slight as possible, Formulas XVII to XXII may be given to E and F.

Actinomycins E_1 and E_2 are formed when isoleucine is added to the culture broth of strains able to produce the actinomycin C complex. Unlike C_3, actinomycin E_1 (Formula XVII) contains one molecule of *N*-methylvaline and one molecule of *N*-methylisoleucine, while actinomycin E_2 (XVIII) has two molecules of *N*-methylisoleucine. Thus the addition of isoleucine to a strain normally producing actinomycin C_3 induces the cell to incorporate into the peptide groups one or two molecules of *N*-methylisoleucine instead of one or two molecules of *N*-methylvaline.

As Schmidt-Kastner has found, the F actinomycins are formed on addition of sarcosine to the culture broth of strains able to produce the actinomycin C complex. Actinomycin F_2 (Formula XIX) may be regarded as deriving from actinomycin C_2 (VI) by the substitution of one molecule of sarcosine for one molecule of proline. The two serpentine lines (XIX) indicate that it is not yet proved whether proline and valine are located in the right peptide group or in the left.



XIX Actinomycin F_2



XX Actinomycin F_1

Actinomycins F_3 (Formula XXII) and F_4 (XXI) may be regarded as derivatives of C_3. Thus actinomycin F_4 may be said to be a C_3 in which one molecule of proline is replaced by one molecule of sarcosine, and F_3 may be said to be a C_3 in which two residues of proline are replaced by two residues of sarcosine.

It should be mentioned that on adding sarcosine to cultures of certain

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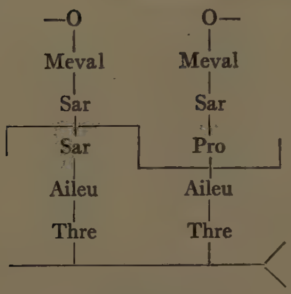
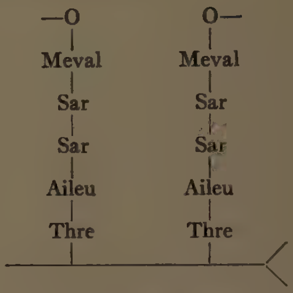
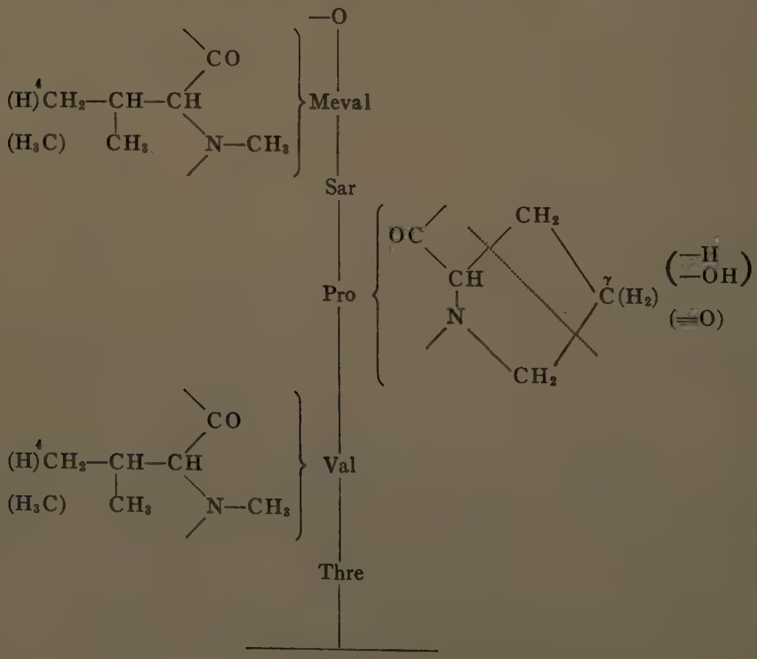
* "Normal actinomycins" are in this case actinomycins that are synthesized by the strains under normal culture conditions, that is, without addition of amino acids.


XXI Actinomycin F_4


XXII Actinomycin F_3

Streptomyces strains Katz and Goss[18] isolated an actinomycin, named actinomycin II, which according to the findings of Johnson and Mauger[19] contains four molecules of sarcosine. It differs from actinomycin F_3 in containing two molecules of valine instead of two molecules of alloisoleucine. Therefore actinomycin II may be considered an actinomycin C_1, in which two residues of sarcosine are substituted for two residues of proline.

If we sum up the observed structural differences among the actinomycins under discussion, we arrive at the following conclusion. All these actinomycins contain the same chromophoric group that we have named actinocin. In all these actinomycins the two carboxy groups of actinocin are bonded to the amino groups of two molecules of threonine. Variations of the structure have been found in three constituents of the peptide groups (Formula XXII): the valine, the *N*-methylvaline, and the proline. In the valine as

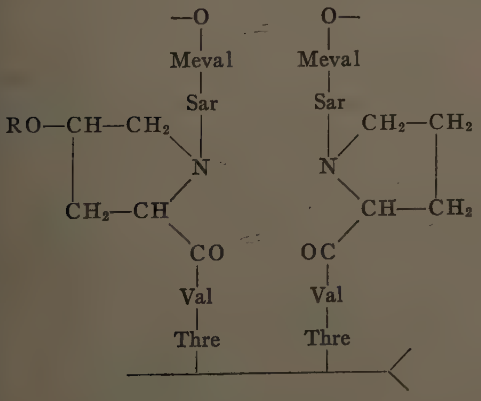

XXIII

well as in the *N*-methylvaline a methyl group can be substituted for a hydrogen atom at carbon atom four, either in only one peptide group or in both. In the proline group (1) a hydroxy group can be substituted for one hydrogen atom at the γ-carbon atom or (2) a carbonyl oxygen can be substituted for two hydrogen atoms at the γ-carbon atom. Finally, one or two molecules of sarcosine can be substituted for one or two residues of proline or, in other words, the $—CH_2—CH_2—$ group (see dashed line in Formula XXIII) of the proline can be replaced by two hydrogen atoms.

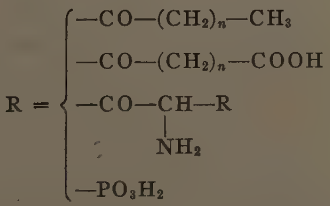
Obviously, there exist still other variations of the peptide groups. In fact, Bossi and his co-workers[21] recently isolated five actinomycins—Z_1, Z_2, Z_3, Z_4, and Z_5—which all contain *N*-methylalanine. Moreover, in our laboratory, J. H. Manegold has isolated an actinomycin that contains only one molecule of threonine in addition to two molecules of valine, two molecules of sarcosine, one molecule of *N*-methylalanine, and an imino acid; it seems to be a derivative of γ-oxoproline. It may be mentioned that, on addition of pipecolic acid to a culture of *Streptomyces* strains, Katz and Goss[18] have produced actinomycins that contained pipecolic acid.

Since Hackmann[22] discovered their carcinolytic activity it has been of great interest whether or not there are differences among the actinomycins in toxicity and in carcinolytic activity. From animal studies it was learned that there are indeed differences in the carcinolytic activity. These differences do not, however, appear to be significant, and it is because of this that we have attempted to obtain derivatives of actinomycins in the hope of finding one or another with a better carcinolytic activity than the actinomycins.

Derivatives of the actinomycins may be obtained in either of two ways: (1) by modifying the peptide part or (2) by modifying the chromophoric group. Thus far we have obtained derivatives, the peptide part of which is modified, only from actinomycins $X_{0\beta}$ and $X_{0\delta}$ (Formula XXIV*a*), which contain hydroxyproline or allohydroxyproline. Since in these two actinomycins the hydroxy group of the hydroxyproline or allohydroxyproline is in a free state, it can be esterified with acids (see R in Formula XXIV). It was thus that we obtained an acetate with acetic acid and a palmitate with



XXIV*a* (RO = OH)



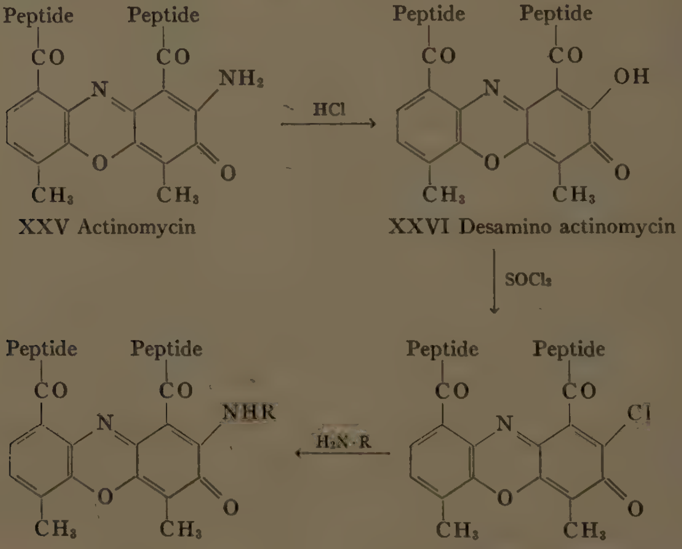
$$R = \begin{cases} —CO—(CH_2)_n—CH_3 \\ —CO—(CH_2)_n—COOH \\ —CO—\underset{NH_2}{\underset{|}{CH}}—R \\ —PO_3H_2 \end{cases}$$

XXIV

palmitic acid. The acetate and the palmitate have less carcinolytic activity than the actinomycins used as starting material. Both derivatives, especially the palmitate, are much more soluble in fat than are the actinomycins, a property that may perhaps be of interest in their medical application.

Esterification of actinomycins $X_{0\beta}$ and $X_{0\delta}$ was also possible with dicarboxylic acids, amino acids, and phosphoric acid. The esters thus obtained (Formula XXIV) have a greater solubility in water. Their carcinolytic activity remains to be checked.

Modifications of the chromophoric group of the actinomycins still are confined to the amino group of the chromophoric group. We have obtained such derivatives in a manner shown by the Formulas XXV to XXVII.



XXV Actinomycin

XXVI Desamino actinomycin

XXVIII

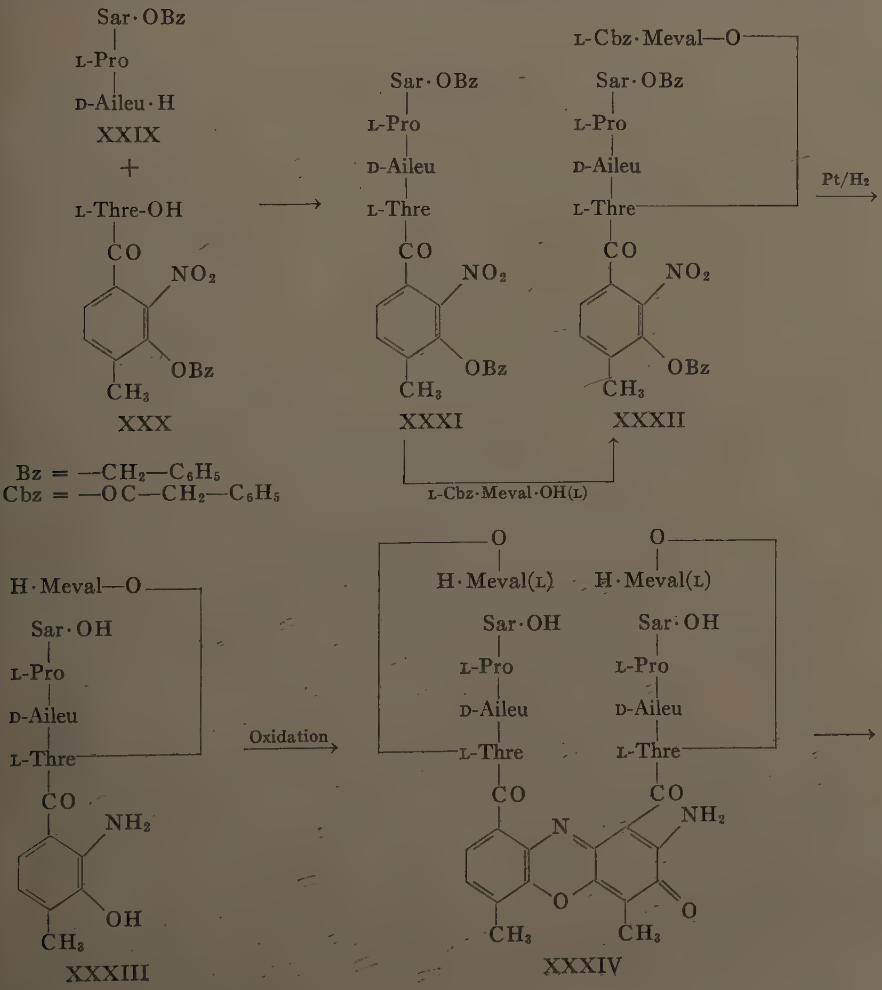
XXVII Chloro actinomycin

If an actinomycin (Formula XXV) is treated under mild conditions (4 hours at 60° C.) with 10 per cent hydrochloric acid, one molecule of ammonia is split off and, in an almost quantitative yield, a desamino actinomycin (XXVI) is formed. During this process the peptide groups remain intact. Contrary to the actinomycins, the desamino actinomycins have neither toxicity nor antibiotic activity. As we have found, chlorine may be substituted for the hydroxy group of the desamino actinomycins by treatment with thionyl chloride. In this way crystallized chloro actinomycins (XXVII) are obtained. If a chloroactinomycin is treated with ammonia, the same actinomycin is recovered that was used as starting material for the preparation of the chloroactinomycin. The chloroactinomycins can also be brought to reaction with primary and secondary amines instead of ammonia. Derivatives of actinomycins are then formed, in which one (Formula XVIII) or both of the hydrogen atoms of the amino group of the chromophore are replaced by alkyl groups or other residues. In this way we have prepared a number of derivatives whose biological activity is still under investigation.

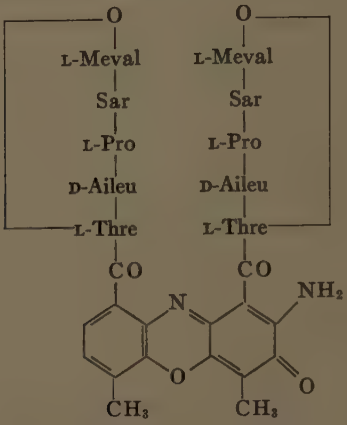
In so far as we can tell, none of these derivatives has a greater carcinolytic activity than do the actinomycins. Whether one or the other has a better therapeutic index remains to be proved.

The aforementioned modifications of the peptide groups and the chromophore are limited by the fact that for the preparation of the derivatives all reactions must be carried out under conditions that do no harm to the peptide groups or to the chromophoric group. From the very beginning, therefore, it was obvious that this limitation would no longer hold if actinomycins and derivatives of them were obtained by synthesis. During the past three years we have been attempting to achieve this, and very recently we were finally successful in synthesizing actinomycin C_3.

The manner of this synthesis is shown by Formulas XXIX to XXXV. The starting materials were the compounds XXIX and XXX. Compound XXX was obtained by coupling L-threonine with the chloride of the 2-nitro-3-benzyloxy-4-methyl-benzoic acid. Compound XXIX is the benzyl ester of



$Bz = -CH_2-C_6H_5$
$Cbz = -OC-CH_2-C_6H_5$



XXXV Actinomycin C_3

the tripeptide D-alloisoleucyl-L-prolylsarcosine. Coupling of the two compounds with the use of dicyclohexylcarbodiimide and triethylamine, according to the method of Sheehan, yielded compound XXXI. Esterification of the threonine-hydroxy group of XXXI with carbobenzoxy-L-*N*-methylvaline was accomplished by means of *N'*,*N*-carbonyl-diimidazol, a reagent first used for esterification by Staab.[23] The compound XXXII thus obtained was separated from the reaction product by chromatography and was then hydrogenated, in order to reduce the nitro group and to split off (a) the two benzyl groups and (b) the carbobenzoxy group.

The reduction product XXXIII was oxidized by means of potassium ferricyanide in phosphate buffer at *p*H 7.3. In this way was obtained a mixture of different products from which, by means of partition chromatography, the compound XXXIV could be separated.

Treatment of compound XXXIV with ethylchloroformate gave, in a yield of about 3 per cent, a compound having the same spectrum, the same R_f values, and the same antibiotic activity as had actinomycin C_3.

There is no doubt that the other actinomycins may be synthesized in the same manner as was actinomycin C_3. Moreover, it now should be possible to produce actinomycins and chromopeptides having chromophoric moieties and peptide moieties other than those of the actinomycins we have at present. Perhaps among such chromopeptides one or another will be found that has better cytostatic activity as well.

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THE CHEMISTRY OF ACTINOMYCIN D AND RELATED COMPOUNDS

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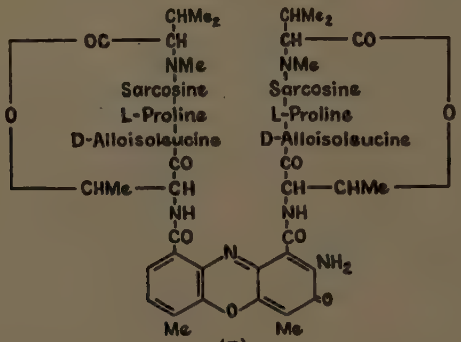
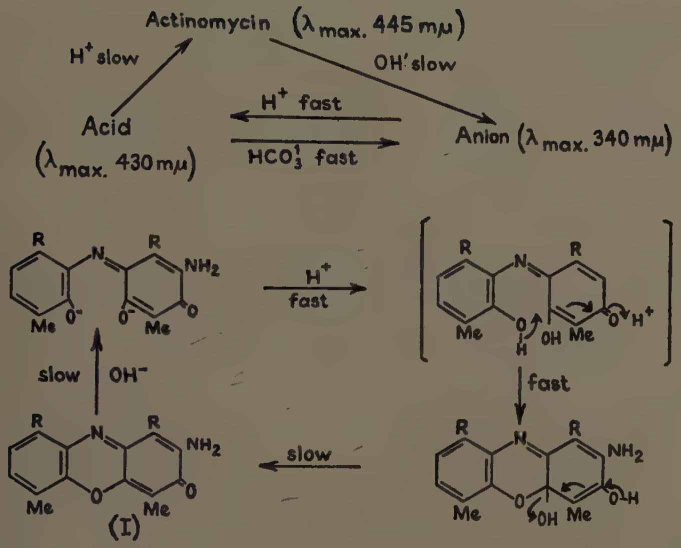
As a result of an extensive series of elegant degradative experiments, the structure of actinomycin C_3 (I, below) was deduced by Brockmann *et al.* in 1956. The red color of the antibiotic is due to the chromophore derived from 2-amino-4,5-dimethylphenoxazin-3-one-1,8-dicarboxylic acid. The acid groups of the chromophore are each attached as an amide to a peptide, L-threonyl-D-alloisoleucyl-L-prolyl-sarcosyl-L-*N*-methylvaline, with the terminal carboxyl group esterified by the hydroxyl group of the threonyl fragment (see Formula I, below).



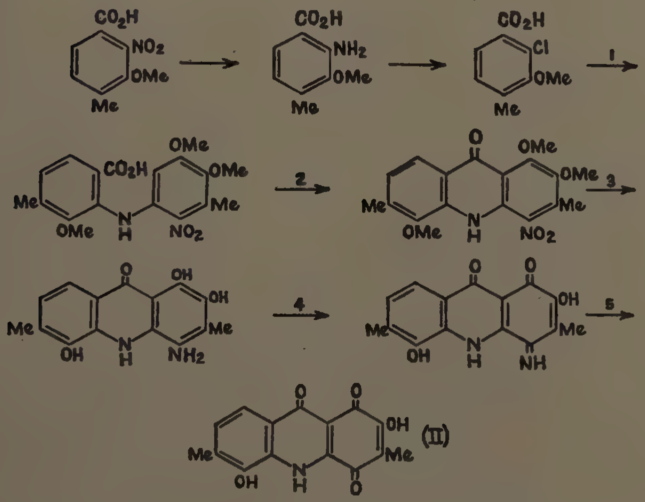
(I)

Several members of the actinomycin family exist, and they differ chemically in the nature of the peptide chains, which are not necessarily identical. The chromophore is common to all actinomycins, and most of the chemical reactions of the actinomycins are associated with the chromophoric grouping. Thus the action of cold dilute alkali causes a marked hypsochromic effect that is associated with the opening of the esterlike oxygen bridge. Acidification of the alkaline solution causes a rapid regeneration of the color, although actinomycin itself is not the primary product. This cyclic process is illustrated and interpreted opposite, identified as *A*. (Angyal *et al.*, 1957).

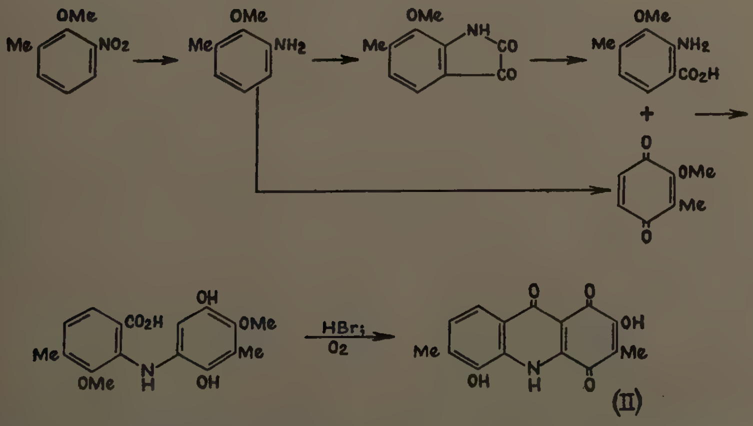
The ultraviolet and visible spectra of actinomycin are not of the usual quinone type (Angyal *et al.*, 1955), but on treatment with hot baryta the peptide groups are split off and the chromophore is rearranged to a true quinone named depeptidoactinomycin by Brockmann and Grübhofer (1950 and 1953) and actinomycinol by Johnson *et al.* (1952). The structure (Formula II) of this rearrangement product, 2,5-dihydroxy-3,6-dimethylacridone-1,4-quinone, was announced by Brockmann and Muxfeldt (1955*a*; cf. 1956*a*) and by Angyal *et al.* (1955) on the same day, and subsequently confirmed by syntheses in Göttingen, German Federal Republic (Brockmann and Muxfeldt, 1955*b* and 1956*b*) and in Nottingham, England (Hanger *et al.*, 1958). Formula *A*, opposite, identified as *B*. is from Brockmann and Muxfeldt, and *B* is from Hanger *et al.*


Actinomycin ($\lambda_{max.}$ 445 mμ)
H⁺ slow
OH′ slow
Acid ($\lambda_{max.}$ 430 mμ)
H⁺ fast
HCO₃′ fast
Anion ($\lambda_{max.}$ 340 mμ)
H⁺ fast
slow OH⁻
fast
slow
(I)

(A)

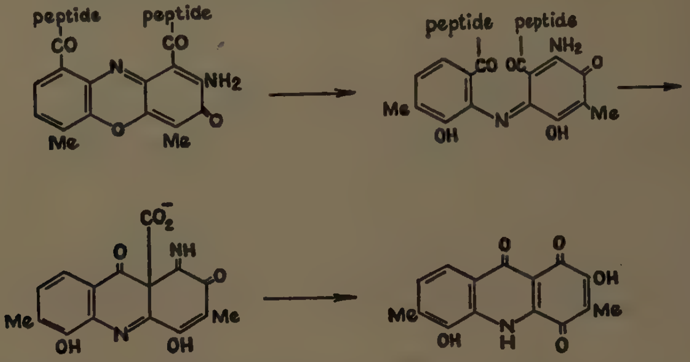


(B)



Reagents: (*1*) 4,5-dimethoxy-3-methyl-2-nitroaniline (Ullmann); (*2*) polyphosphoric acid (*3*) H_2—Ni; HBr. (*4*) O_2; (*5*) HBr—AcOH.

The formation of actinomycinol is a remarkable rearrangement involving several stages, which may be given as follows:



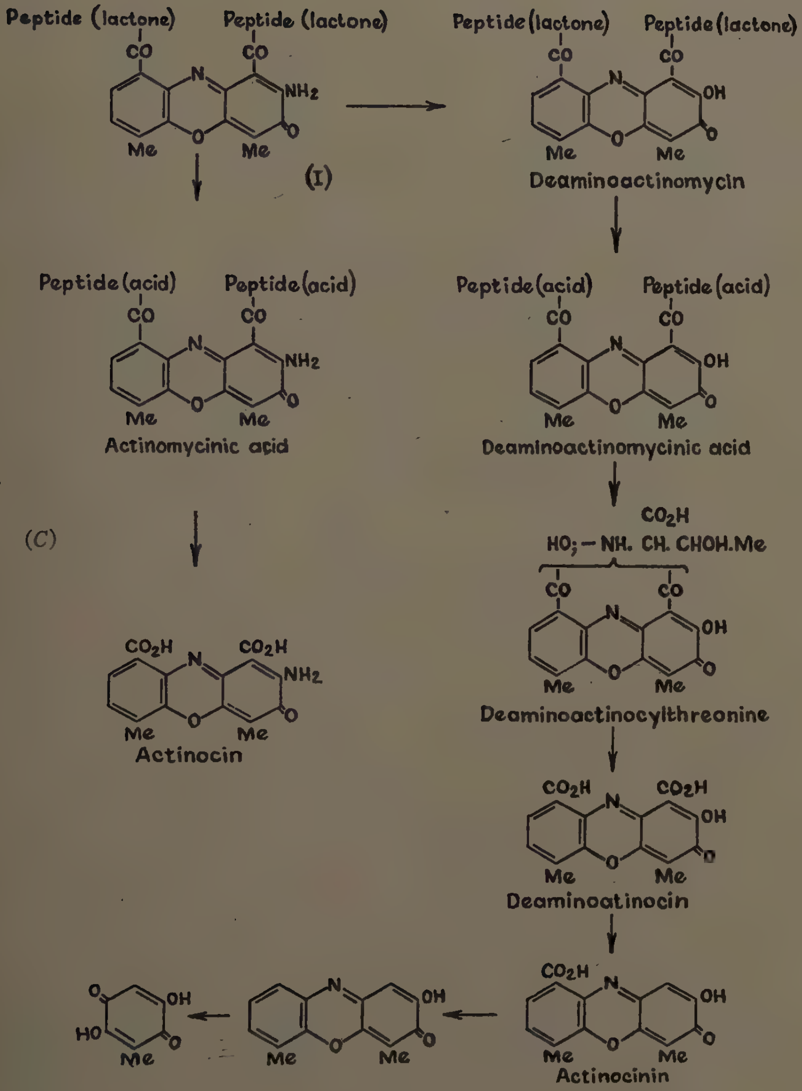
Acid hydrolysis of actinomycin C_3 gave a series of degradation products, and the determination of the structures of these products (Brockmann and Muxfeldt, 1956*c*; Brockmann and Gröne, 1956) led to the recognition of the phenoxazone chromophore of the actinomycins (opposite, identified as *C*).

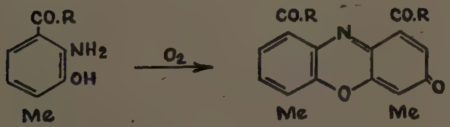
The phenoxazinone nucleus can arise from the oxidative coupling of peptides derived from 3-hydroxy-4-methylanthranilic acid (Johnson, 1956). Such a condensation has been achieved *in vitro* by oxidation of *N*-(3-hydroxy-4-methylanthraniloyl)glycine methyl ester (Formula III), either in air (Brockmann and Muxfeldt, 1956*d*) or in a buffered solution of potassium ferricyanide (Hanger *et al.*, 1958). The formation of the insect pigment xanthommatin from 3-hydroxykynurenin also is a reaction of this type (Butenandt *et al.*, 1954 and 1955) (opposite, identified as *D*).

In the work of Bullock and Johnson (1957*b*) on actinomycin D (or actinomycin IV; Vining and Waksman, 1954) we confirmed the presence of the 2-amino-4,5-dimethylphenoxazin-3-one-1,8-dicarboxylic acid chromophore by demonstrating the similarity of the light absorption of actinomycin D in neutral, acid, and alkaline solutions to the light absorptions of all the other actinomycins (Roussos and Vining, 1956), and by showing also the formation of actinomycinol (Formula II).

Amino acid analysis of actinomycin D gave values that suggested that there were two molecules each of L-threonine, D-valine, L-proline, sarcosine, and L-*N*-methylvaline in the antibiotic; thus the D-alloisoleucine of actinomycin C_3 was replaced by D-valine. However, in a molecule such as that shown in Formula I, which contains two separate peptides, there is the possibility that they are not identical and, consequently, the results of normal peptide degradation must be interpreted with caution. Thus the isolation of a peptide degradation product does not in itself warrant the assumption that it is derived from both peptides. A method therefore was devised whereby the two peptides might be separated: the actinomycin was oxidized with alkaline hydrogen peroxide (Bullock and Johnson, 1957*a*). The two peptide-containing fragments so obtained can be separated by partition between ethyl acetate and water and purified by countercurrent distribution. Each prod-

(C)

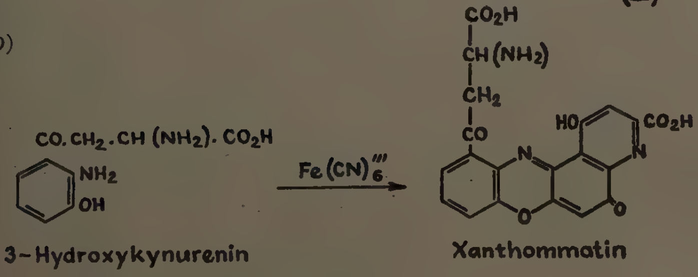




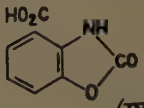
(R = OMe, — NH. CH2. CO2Me)

(III)

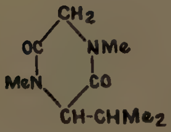
(D)



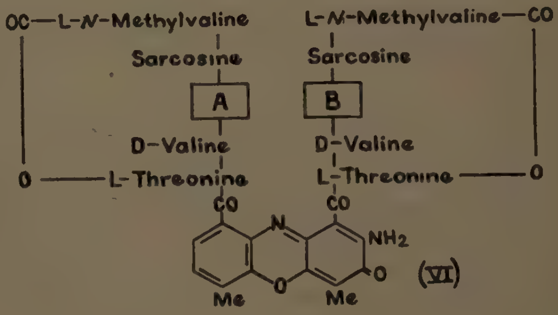
uct contained all five of the constituent amino acids and in addition a nonpeptide fragment. In the first product, the nonpeptide fragment was 7-methylbenzoxazolone-4-carboxylic acid (Formula IV), which was confirmed by synthesis; in the second, it was oxalic acid. The order of the amino acids in the peptides was determined with standard methods of peptide analysis; mention also may be made here of the formation of volatile diketopiperazines, for example, sarcosyl-*N*-methylvalyl (Formula V), by pyrolysis. The structure of actinomycin D thus was determined as that shown in Formula I: with D-alloisoleucine residues replaced by D-valine (Bullock and Johnson, 1957*b*).



(IV)



(V)



(VI)

Katz and Goss (1958 and 1959) have studied the effect of adding various amino acids to the culture medium of *Streptomyces antibioticus* and, in particular, have found that the addition of sarcosine stimulates the production of actinomycins II and III, which are normally trace components. Through the courtesy of Waksman and of Katz, my associate and I have been able to examine these concentrates and have isolated pure actinomycins II and III (Johnson and Mauger, 1959). An amino acid analysis of the acid hydrolysis products of these actinomycins showed that actinomycin III contained three sarcosine residues and only one proline, whereas actinomycin II contained no proline and four sarcosine residues. The actinomycins II, III, and IV thus constitute a series (Formula VI) in which proline and sarcosine are interchangeable. Brockmann and Manegold (1958) have shown that actinomycins I and V differ from IV in that the proline has been replaced partially by 4-hydroxyproline and 4-oxoproline, respectively.

Actinomycin III is presumed to be a mixture of two isomers formed by the random oxidative coupling of the two 3-hydroxy-4-methyl-anthraniloyl peptides, that is, actinomycin II-type and actinomycin IV-type peptides, and some experimental evidence suggests that this is the case (Johnson and Mauger, 1959). A similar situation obtains with actinomycins C_2 and C_{2a} (Brockmann and Franck, 1960), where again the two peptide chains are not equivalent.

There is evidence suggesting that L-pipecolic acid also is incorporated into the actinomycin molecule in place of L-proline (Katz and Goss, 1959); an examination of such concentrates is in progress.

Acknowledgment

It is a pleasure to acknowledge the contributions of my colleagues, particularly Alexander Todd, E. Bullock, W. G. Hanger, W. C. Howell, A. B. Mauger, and L. C. Vining, to these studies on the chemistry of the actinomycins.

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MECHANISM OF ACTION OF ACTINOMYCIN D IN MICROBIOLOGICAL SYSTEMS

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Since their discovery in 1940 by Waksman and Woodruff,[1] the actinomycins have been shown to be characterized by marked cytotoxicity in several biological systems. Members of this group of antibiotic substances effectively inhibit the growth responses of a wide variety of Gram-positive bacteria and, to a limited extent, of Gram-negative bacteria and fungi. An extensive literature[2-4,5] that includes the reports presented in this monograph testifies to the production of significant selective antineoplastic activity by the actinomycins in man, experimental animals, and cell lines in tissue culture. This broad spectrum of toxicity coupled with the nature of elicited cellular antagonism gives the actinomycins a relatively rare position among antibiotic substances. A review of the salient reported data with additional observations relating to the effects of actinomycin D in microorganisms will be presented in this report.

Preliminary observations on the mechanism of action of actinomycin D in microorganisms were first made by Foley and Eagle,[6] who used the specific bioassay organisms *Lactobacillus arabinosus*, *Lactobacillus casei*, *Leuconostoc citrovorum*, *Lactobacillus fermenti*, and *Streptococcus faecalis*. In each instance the test organism had an exogenous requirement for pantothenic acid, and the inhibition by actinomycin D could be reversed competively only by the presence of pantothenic acid in the culture medium. Effective noncompetitive reversal was observed with L-methionine, orotic acid, adenine, pyruvate, and oxalacetate. Inhibition analysis results were compatible with a hypothesis that this antibiotic (in these nutritionally pantothenate-requiring strains) interfered with pantothenic acid-dependent reactions concerned with the biosynthesis or the utilization of amino acids, or both.

By contrast, it was observed in my laboratory[7] that actinomycin D at low concentrations completely inhibited the growth response of *Bacillus subtilis* ATCC 6051 either in a synthetic medium containing no added growth factors or in a medium containing excess pantothenic acid. No reversal of actinomycin D growth inhibition against *B. subtilis* was achieved with any, whether single or mixed, of a large number of chemical agents that included β-alanine, potassium pantoate, nucleic acid, purine, and pyrimidine bases, nucleosides and nucleotides, the members of the B-vitamin group, the amino acids found in casein hydrolysate, succinate, fumarate, pyruvate, citrate, and orotic acid. Neither did actinomycin D inhibit the synthesis of pantothenic acid by appropriate enzymes or alter the coenzyme A content of cells of *B. subtilis*. The acetylation of sulfanilamide by cell-free extracts of *B. subtilis*, a coenzyme A-linked reaction, was also insensitive to actinomycin D.

The effects of actinomycin D on the growth response of *Saccharomyces cerevisiae*, with β-alanine as growth-limiting substrate, are listed in TABLE 1. The basal medium was that of Billen and Lichstein,[8] with supplements added as indicated. For optimal growth of this strain, 2 μg./ml. of β-alanine were required. In medium containing this amount, actinomycin D in concentrations of up to 50 μg./ml. did not inhibit or stimulate growth but, when β-alanine was present in suboptimal concentrations (0.5 to 1.0 μg./ml.), it was

TABLE 1
RELATIONSHIP BETWEEN β-ALANINE AND ACTINOMYCIN D INHIBITION OF *SACCHAROMYCES CEREVISIAE*

β-Alanine (μg./ml.)	Actinomycin D (μg./ml.)	Growth inhibition (%)*
0.5	0	0
0.5	1.5	0
0.5	6.0	36
0.5	12	73
0.5	25	80
0.5	35	86
0.5	50	100
1.0	6	0
1.0	12	18
1.0	25	39
1.0	35	53
1.0	50	71
2.0	35	0
2.0	50	0

* Calculated after 24 hours at 30° C.

TABLE 2
RELATIONSHIP BETWEEN PANTOTHENATE AND ACTINOMYCIN D INHIBITION OF *SACCHAROMYCES CEREVISIAE*

Pantothenate (μg./cc.)	Actinomycin D (μg./ml.)	Growth inhibition (%)*
0.01	50	38
0.01	35	21
0.01	20	17
0.01	12	17
0.1	50	33
0.1	35	26
0.1	20	11
0.1	12	2
1.0	50	30
1.0	35	12
1.0	20	6
1.0	12	3

* Calculated after 24 hours at 30° C.

an effective inhibitor of growth. The amount of actinomycin D producing half-maximal growth in medium containing 0.5 and 1.0 μg./ml. of β-alanine was 9.5 μg./ml. and 32 μg./ml., respectively. TABLE 2 lists the effects of actinomycin D on the growth response of *S. cerevisiae*, with pantothenate as growth-limiting substrate. Here the growth inhibition by actinomycin D was independent of the concentrations of pantothenate added to the medium. Relatively high concentrations of actinomycin D were necessary to produce a partial inhibition of growth. Complete inhibition was not observed with as much as 50 μg./ml. of actinomycin D. Several compounds were tested for ability to reverse the growth inhibition produced by actinomycin D (10 μg./ml.) in medium containing suboptimal growth concentrations of β-alanine (0.5 μg./ml.). These were tested at final concentrations of 0.01 *M*, except as indicated below, and included the following: adenine, guanine, hypoxanthine, xanthine, uracil, inosine, 4(5)-amino-5(4)-imidazole-carboxamide, ureisosuccinic acid, vitamin-free casein hydrolysate (0.5 per cent), thiamine (10^{-4} *M*), riboflavin (10^{-4} *M*), biotin (1 μg./ml.), folic acid (10^{-6} *M*), pyridoxine (10^{-4} *M*), nicotinic acid (10^{-4} *M*), inositol (10^{-4} *M*), and B_{12} (1 μg./ml.). No significant reversal of growth inhibition was observed with any of these compounds.

TABLE 3

EFFECT OF ACTINOMYCIN D UPON ENTRY OF AMINO ACIDS IN MICROORGANISMS*

Organism	Actinomycin D (μg./ml.)	Amino acid	Entry (μg./100 mg.)	Inhibition (%)
Streptococcus faecalis 8043	0	glutamate	564	—
	10	glutamate	201	65
	0	lysine	290	—
	10	lysine	303	0
Bacillus subtilis 6051	0	glutamate	470	—
	10	glutamate	162	66
	0	lysine	105	—
	10	lysine	111	0
Saccharomyces cerevisiae	0	glutamate	388	—
	10	glutamate	376	0
	0	lysine	134	—
	10	lysine	126	0

* Methods used have been described by Gale.[9]

Unfortunately, actinomycin D could not be tested against pantothenate-requiring strains of *B. subtilis* because of their unavailability. Since wild-type strains of *Escherichia coli* are insensitive to this antibiotic, it is perhaps of lesser significance that the pantothenate-requiring mutants of this organism also were not inhibited by actinomycin D.

A comparison of the influence of actinomycin D on the rate of entrance and assimilation of some amino acids in *Str. faecalis*, *B. subtilis*, and *S. cerevisiae* is given in TABLE 3. Although the assimilation of both glutamate and lysine was unimpaired in the three organisms, actinomycin D effectively depressed the entrance of glutamate into *Str. faecalis* and *B. subtilis*, while it had no effect on the entrance of lysine. It has been pointed out by Gale[9] that glutamate entrance requires an energy source (active transport mechanism), whereas lysine is able to diffuse freely in, as well as out of, microbial cells. The failure to alter the rate of entrance of glutamate into *S. cerevisiae* may be attributed to differences in yeast cell wall structure and permeation systems between this organism and *Str. faecalis* and *B. subtilis*.

It has been reported previously[10] that actinomycin D suppressed the assimilation of ammonia and completely inhibited the formation of certain inducible enzymes in *B. subtilis*. Furthermore, the addition of growth-inhibiting concentrations of this antibiotic to logarithmic phase cultures of *B. subtilis* immediately halted the synthesis of protein and ribonucleic acid (RNA), whereas desoxyribonucleic acid (DNA) synthesis continued at the same rate as in the control cultures[11] (FIGURE 1). The only other reported example of a similarly disproportionate production of DNA has been observed by Pardee and Prestidge[12] in *E. coli* inhibited by β-2-thienylalanine. Since β-2-thienylalanine caused no growth inhibition of logarithmic growing cells of *B. subtilis*, and since *E. coli* was insensitive to actinomycin D, comparative studies were not possible. Efforts to restore a balanced DNA, RNA, and protein synthesis in the presence of actinomycin D through the addition of several compounds, including β-alanine and pantothenic acid, have proved unsuccessful.

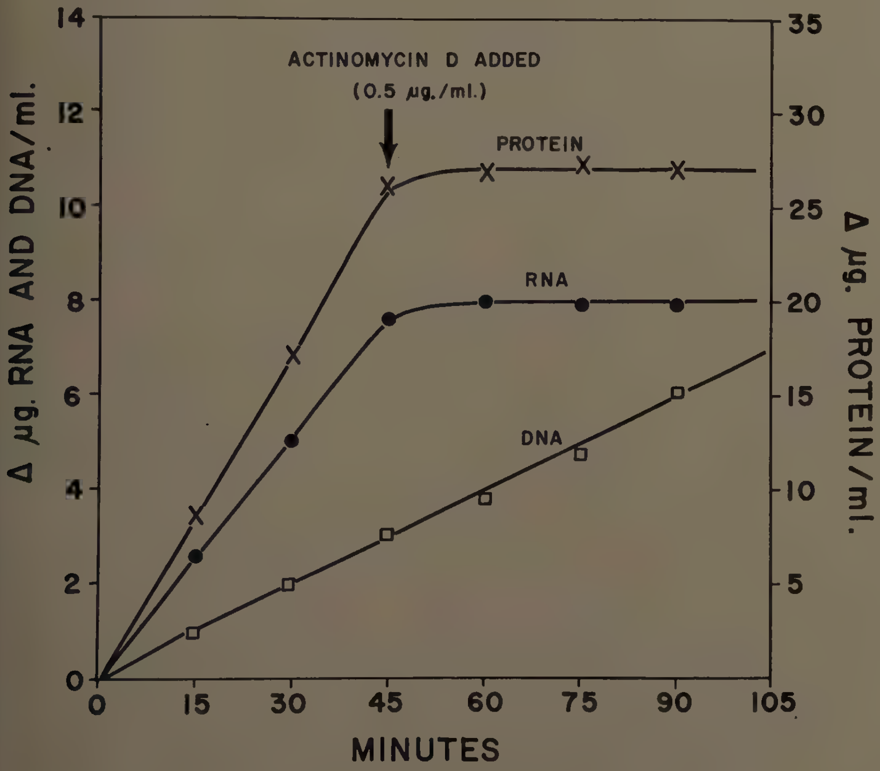

FIGURE 1. Effect of actinomycin D on synthesizing of protein, ribonucleic acid, and desoxyribonucleic acid by *Bacillus subtilis*.

Since the increasing number of human infections from virulent penicillin-resistant staphylococci has become of widespread concern, an *in vitro* survey of the sensitivity of these organisms to actinomycin D was undertaken. Tested by a standard paper-disc agar-diffusion method, each of 137 penicillin-resistant coagulase-positive strains of staphylococci (isolated from clinical infections) was equally sensitive to actinomycin D. Neither the initial level of penicillin resistance nor the *in vitro* increases of actinomycin D applications altered this sensitivity. Preliminary *in vivo* studies of mice inoculated with virulent staphylococci have shown encouraging levels of protection with nontoxic doses of actinomycin D.

Analysis of several actinomycin D-resistant strains of *B. subtilis*, obtained by the gradient plate technique, disclosed that these organisms had lost the ability to form spores in a medium containing actinomycin D as well as in several drug-free media.[13] Some degree of specificity was attached to this asporogeny, associated with the development of resistance, since sulfanilamide- or A-Methopterin-resistant mutants of *B. subtilis* were able to sporulate.

Discussion

The investigative protocols have revealed that growth inhibition caused by actinomycin D is competitively altered by the presence of pantothenic acid in several organisms having exogenous requirements for this vitamin, but not in organisms capable of pantothenate-independent growth. Other examples of similar differences in drug action have been found when nutritionally independent, sensitive strains were compared with nutritionally dependent organisms. The lack of adequate explanation and the more than isolated occurrence of this type of substrate-drug action relationship generously support the opinion that a complete understanding of the mechanisms involving such divergencies in drug action is of fundamental importance to a knowledge of the modes of drug toxicity.

Although certain speculative theories about the mechanism of action of actinomycin D may be provided, several essential questions remain to be answered before the ultimate explanation is found. What cellular component(s) interact with this agent? What is the nature of any binding? What is the primary site of drug action? Indeed, does actinomycin D penetrate into the cell at all or does its biological activity manifest itself on or at the cell surface? With what specific point(s) in reactions leading to the synthesis of cellular protein or ribonucleic acid does actinomycin D interfere? To what extent do structural modifications in the chromophore or phenoxazone moieties or in both alter specific biological activities? Investigations pertinent to some of these problems are in progress.

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Part II. Pharmacological and Experimental

THE TOXICITY OF ACTINOMYCIN D*

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The high toxicity of the actinomycins in mammals was first described after the isolation of the A complex from *Streptomyces antibioticus* by Waksman and Woodruff,[1] whose early work directed attention to what appeared at first a selective atrophy of the mammalian spleen.[2,3] These investigators also showed that doses not greatly exceeding the LD_{50} killed, within less than 24 hours. Somewhat more delayed fatalities were caused by lower single doses or by repeated daily injections of small fractions of the acute LD_{50}. Anorexia, diarrhea, disturbances of locomotion, and weakness were features of chronic poisoning. Hackmann's later studies of the C complex showed that the spleen was not the only susceptible lymphoid tissue: thymus and lymph nodes were equally or, possibly, more sensitive sites of damage.[4,5] He also reported mitotic inhibition in the crypts of Lieberkühn, as well as degeneration and desquamation of the intestinal epithelium. Untoward clinical effects such as stomatitis, nausea, and diarrhea were considered by him to be related manifestations of gastrointestinal damage.

Our present interest in these compounds stemmed primarily from the description given by Hackmann and by Schmidt-Kastner of the biosynthetic actinomycin F_1 (KS_4).[6,7] Since F_1 had antitumor activity but was significantly less toxic than earlier actinomycins, it seemed worthwhile to learn whether or not it might be distinguished by other qualitatively different pharmacological effects. For a more meaningful comparison, we decided to become familiar first with the toxic properties of actinomycin D[8,9] in different laboratory animal species. This is the subject of the present communication, which extends the earlier observations of other workers.[10,11]

Methods

Actinomycin D was dissolved by first warming (steam bath) in propylene glycol (U.S.P.) and then diluting with water. All solutions for use in mice and rats had a constant propylene glycol concentration, 10 per cent in water v/v; injections were given in the constant volume of 0.01 ml./gm. Solutions for use in dogs had much lower final concentrations of propylene glycol; 0.02 ml./kg., a negligible quantity, was the maximal daily amount given. The other procedures have been described before.[12]

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Results

Toxicity in mice and rats. TABLE 1 lists the LD_{50} in mice and rats given single or repeated daily doses by different routes. The agent was much less potent by oral intubation than by parenteral injection. Subcutaneous doses had about one half the toxicity of intraperitoneal doses. Toxicity was almost precisely cumulative: the LD_{50} of multiple doses was essentially equal to that of single doses divided by the number of successive daily doses given.

FIGURE 1 shows the fate of rats receiving intraperitoneal and subcutaneous doses. Except in the animals given single intraperitoneal doses of 0.63 or 0.31 mg./kg., most fatalities occurred within less than 48 hours after injection (or within less than 48 hours after the last of five successive daily doses). Similarly short survival times were seen in rats given lethal doses by vein or by mouth, as well as in mice treated by all routes.

TABLE 1

TOXICITY OF ACTINOMYCIN D IN MALE HA/ICR MICE (20 TO 30 GRAMS) AND MALE CFN WISTAR RATS (200 TO 300 GRAMS)*

Species and route	No. success. daily doses	LD_{50}† (mg./kg./day)	19/20 confidence limits† (mg./kg./day)	S†
Mice				
I.P.	1	0.76	0.53–1.08	1.8
S.C.	1	1.40	1.14–1.72	1.3
Oral‡	1	13.0	9.5–17.7	1.7
I.P.	5	0.14	0.12–0.17	1.2
S.C.	5	0.21	0.17–0.26	1.3
Rats				
I.V.	1	0.46	0.37–0.57	1.3
I.P.	1	0.40	0.21–0.74	3.0
S.C.	1	0.80	0.63–1.01	1.3
Oral‡	1	7.2	5.0–10.3	1.6
I.P.	5	0.088	0.067–0.12	1.4
S.C.	5	0.16	0.12–0.21	1.3

* Doses given in the constant volume of 0.01 ml./gm.; animals observed for 14 days after the final injection.

† Calculated according to Litchfield and Wilcoxon.[13]

‡ By intragastric intubation in animals starved overnight.

The signs of toxicity were like those in animals given actinomycin A.[3] Lethal doses were without effect for five to six hours. Thereafter diarrhea began, and by the end of 24 hours it was prominent even in rats given sublethal doses. With the exception of those rats injected with the lower intraperitoneal doses, fatally intoxicated animals were greatly depressed and dyspneic if still surviving after 24 hours. They became moribund, with slow labored breathing, before they died.

Abundant quantities of bloody cloudy fluid were found in the thoracic and abdominal cavities of rats that survived more than 24 hours after intraperitoneal doses (these effusions were not seen in mice). Those rats that succumbed during the second week developed extensive adhesions among the major abdominal viscera and deposits of fibrinlike material on the mesenteries. Since these signs of peritonitis were absent in animals injected by any of the three other routes, the peritoneal reaction was deemed the consequence of local contact damage. This was supported by the observation that subcutaneous doses produced within 48 hours edema and necrotic changes at the sites of inoculation. Eventually the overlying skin became indurated and hairless and developed eschars. Subcutaneous damage was seen in both rats and mice after injection of concentrations as low as 6 γ/ml. Such high capacity for local damage *in vivo* is perhaps not surprising in view of the outstanding cytotoxicity of actinomycin D in mammalian cells *in vitro*.[14] We consider it likely that the added burden of peritonitis was the chief cause of the delayed deaths in rats given the lowest fatal intraperitoneal doses (FIGURE 1).

MALE RATS: ACTINOMYCIN D													
DOSE mg/kg /day		INTRAPERITONEALLY						SUBCUTANEOUSLY					
	MORTALITY	DAY OF DEATH						MORTALITY	DAY OF DEATH				
		1	2	3-4	5-7	8-10	11-14		1	2	3-4	5-7	8-10
		SINGLE DOSES											
5.0	3/3	●●●						3/3	●●●				
2.5	8/9	●●●●●●●●						6/6	●●●●●●				
1.25	8/9	●●●●●●	●●					6/6	●●	●●●●			
0.63	6/9		●●●●	●	●			1/6		●			
0.31	6/9				●●	●●	●●	0/6		●			
0.16	0/6												
		DAILY DOSES X 5											
0.5	3/3		●●	●				6/6			●●●●●●		
0.25	6/6			●●●●●●				6/6			●	●●●●●	
0.13	6/6			●●	●●●●			1/6					●
0.06	0/6							0/6					

FIGURE 1. Fate of rats given intraperitoneal and subcutaneous doses.

Pathology in rats. In order to avoid the complications of peritonitis we employed subcutaneous injections for studying the pathological effects of actinomycin D in rats. As shown in FIGURES 2, 3, and 4, two doses were used. The higher, 0.2 mg./kg./day, was somewhat above the LD_{50} (as previously determined in the toxicity studies listed in TABLE 1); it was given to 24 rats from which groups of six were selected at random on days 2 and 4. The remaining 12 received a fifth dose and all died between days 5 and 7. The lower dose, 0.1 mg./kg./day, was also given to 24 rats. Groups of six were selected on days 7 and 9; the remainder survived. With each experimental group, 6 controls were selected that had been treated identically except for the omission of actinomycin D from the injection fluids. FIGURE 2 shows the uniform

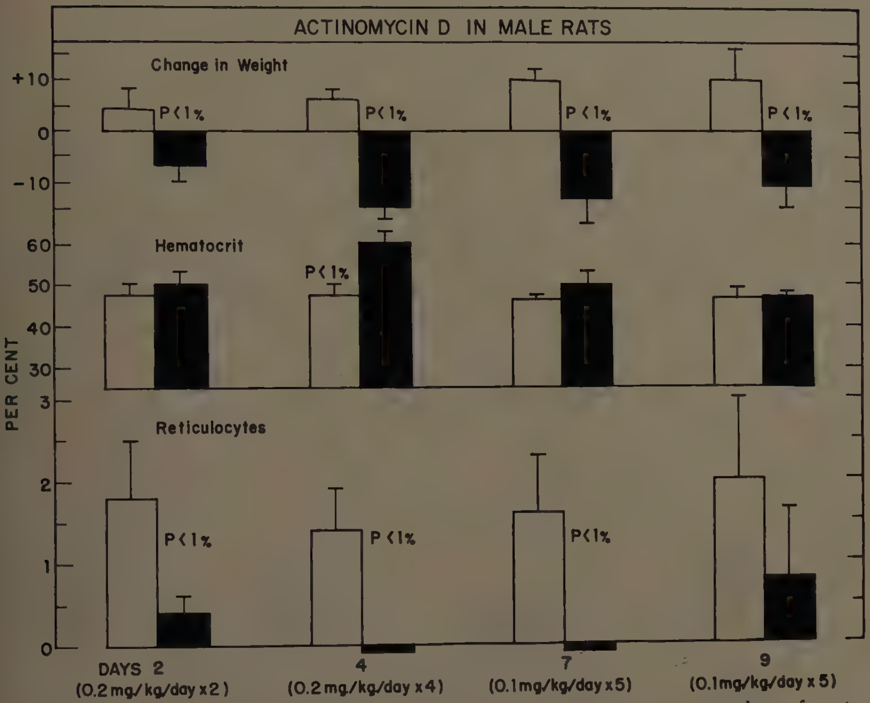

FIGURE 2. See text for description of experiment. Open bars are mean values of control animals; black bars, of the experimental animals. The height of the T symbol above or below the bars represents the value of one standard deviation. P values were calculated by the conventional *t* test for significance.

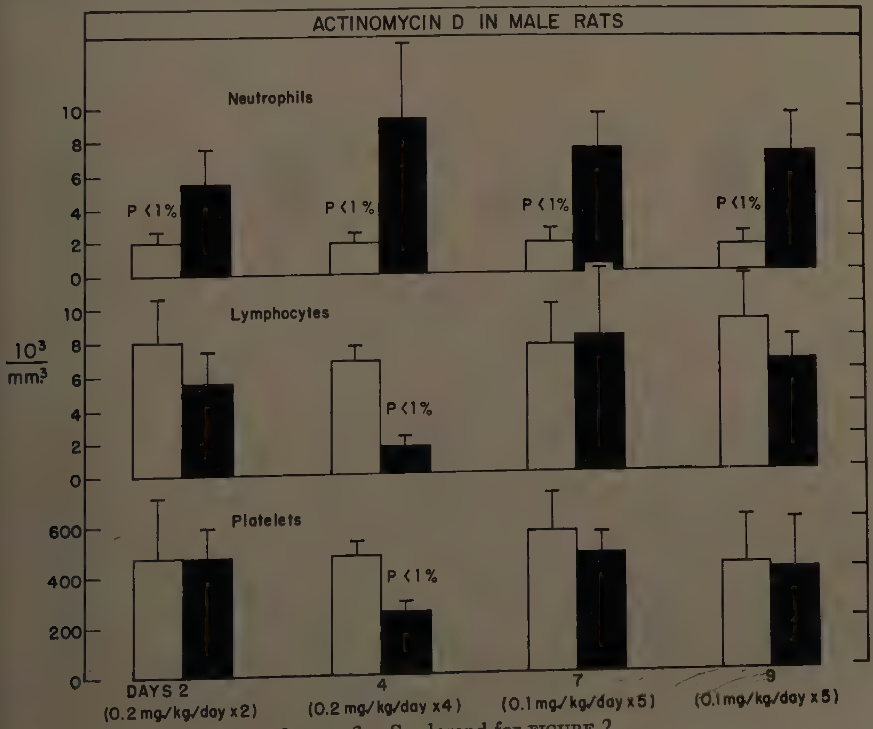

FIGURE 3. See legend for FIGURE 2.

weight loss of all experimental groups. It also shows that lethal doses caused marked rises in hematocrit values. The losses in weight and the hemoconcentration indicated severe dehydration, probably due to anorexia and diarrhea.

Reticulocytopenia was another uniform change shown in FIGURE 2. Effects in other blood cells are depicted in FIGURE 3. All experimental groups had marked neutrophilic granulocytosis, but only fatally poisoned animals developed significant lymphopenia and thrombocytopenia. FIGURE 4 shows the response of different lymphoid tissues. The data support Hackmann's earlier observations[5] with actinomycin C: the lymphocytotoxic effects of the actinomycins are not limited to the spleen.

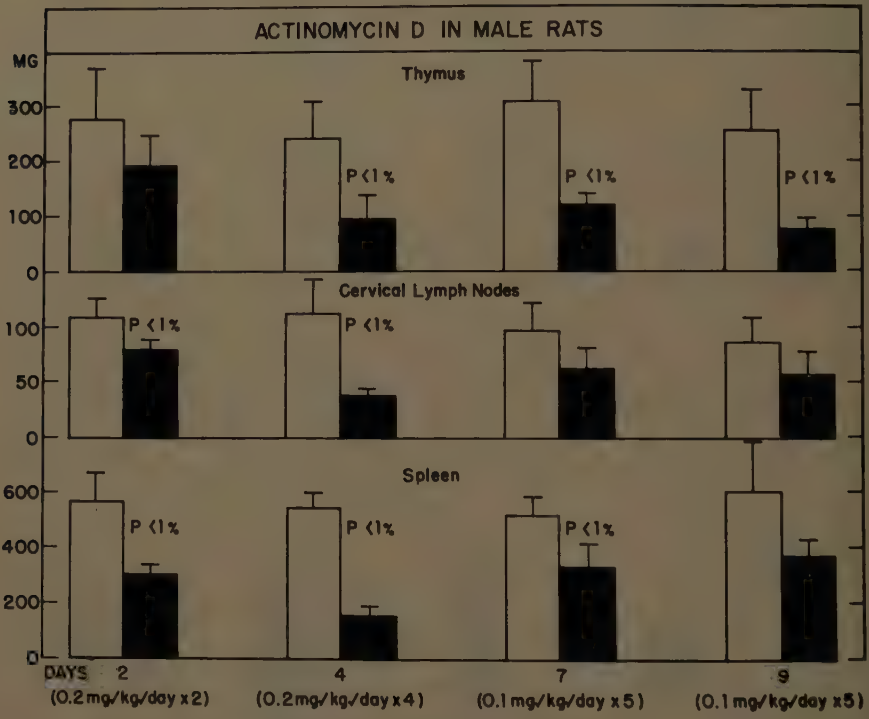

FIGURE 4. See legend for FIGURE 2.

Microscopic studies were done with three animals from each of the same experimental groups. The day-4 animals had the most severe changes, and only they will be described in detail. The effects seen on the other days were consistent with the day-4 results. The lymphoid tissues of the day-4 rats were greatly altered. The nodes showed karyorrhexis, loss of follicular pattern, and decreased numbers of the more mature lymphocytes. The thymi were involuted and severely karyorrhectic. The follicles of the spleen were small and also showed karyorrhexis. The red pulp was uniformly contracted. The content of nucleated cells in the sternal bone marrow was reduced 50 to 80 per cent. Karyorrhexis was also evident in these cells. The epithelium of the small and large intestine was extensively damaged and atrophic. There was swelling of cells, loss of nuclear polarity, ectasia and, in the basal regions of the small intestine, karyorrhexis. Submucosal edema and congestion were prominent. There were no lesions in any of the other abdominal and thoracic viscera.

Two other effects were seen. The subcutaneous sites of injection were edematous and hemorrhagic. Degenerative changes, inflammation, and necrosis were present in adjacent fat and muscle. In the day-9 rats there was early ulceration of the overlying skin. Testicular abnormalities were found in three of the six day-6 and day-9 rats. These varied from isolated giant multinucleated epithelial cells to foci of necrosis in the spermatogenic epithelium and complete spermatogenic arrest.

Effects of adrenalectomy. Since the lymphocytotoxic effects of actinomycin D occur in animals that are affected by the stresses of diarrhea, dehydration, weight loss, and severe intestinal damage, it is necessary to inquire whether they are direct actions or whether they are mediated indirectly through arousal of the pituitary-adrenal axis.[15] To test this, pairs of animals from each of the four groups described in TABLE 2 were killed and analyzed on the same day; the totals shown in the table were accumulated by repetition of the entire experiment. (In preliminary studies the dose used in TABLE 2 had been shown to satisfy the requirement that it be tolerated by sensitive adrenalectomized animals, while causing significant lymphoid changes in intact animals.)

TABLE 2 shows that significant lymphoid changes were produced in both experimental groups. They were possibly greater in the intact animals, in which the effects of actinomycin D could have been enhanced by adrenal cortical stimulation. Histological study of representative animals among both adrenalectomized and intact rats treated with the agent revealed the kinds of changes in lymphoid tissues that have been described above: for example, involution of thymus and reduction in size of splenic follicles, as well as marked karyorrhexis in their central regions. The failure to find a significant hypertrophy of the adrenals of treated intact animals (TABLE 2) was unexpected, since this change had previously been seen by Hackmann in strain G rats given actinomycin C.[5]

The results of this experiment provide convincing evidence of the direct action of actinomycin D on lymphoid cells. In this respect the agent closely resembles both nitrogen mustards and ionizing radiations, which have been the only toxic agents known to induce lymphoid damage in adrenalectomized animals.[15]

Toxicity in dogs. TABLE 3 describes the fate of 22 dogs receiving intravenous doses of actinomycin D. As previously seen in mice and rats, the cumulative toxicity of the agent is evident in a comparison of the effects of single and multiple doses. All of the fatally intoxicated animals and most of the survivors had diarrhea. This appeared within 24 hours in the pair given 200 γ/kg. and after three to four days in the others. The stools became bloody in most of the animals that died. Anorexia and weight loss began during the second or third day after 100 γ/kg. or 20 γ/kg./day, and at later times following lower doses. Emesis was another disturbance more pronounced after the high single doses than after the lower repeated daily doses. It did not appear until at least two to three hours after injection. Four of the dogs receiving repeated daily doses had swollen inflamed legs due undoubtedly to local effects at sites of injection.

TABLE 2

EFFECT OF ADRENALECTOMY ON THE RESPONSE OF LYMPHOID ORGANS TO ACTINOMYCIN D IN MALE RATS*

Experimental Groups Received 0.5 μg./kg. Subcutaneously on Day 0

Group	No. animals	Initial wt. (gm.)	Change in wt. (%)	Wet weight (mg.)			
				Thymus	Spleen	Cervic. lymph nodes	Adrenal
Day 3							
Intact control	10	257 ± 21	3 ± 2	244 ± 44	626 ± 141	88 ± 27	27.3 ± 4.6
Experimental	10	256 ± 28	−11‡ ± 2	128‡ ± 57	212‡ ± 57	40‡ ± 8	31.5 ± 5.9
AdX† control	10	244 ± 19	3 ± 4	387 ± 86	516 ± 46	95 ± 18	
Experimental	10	251 ± 10	−6‡ ± 4	233‡ ± 69	282‡ ± 29	55‡ ± 12	
Day 4							
Intact control	4	257 ± 28	8 ± 0	246 ± 61	643 ± 131	89 ± 11	30.3 ± 0.8
Experimental	4	248 ± 25	−13‡ ± 7	103¶ ± 27	201‡ ± 53	40‡ ± 11	31.2 ± 5.7
AdX† control	4	245 ± 15	7 ± 2	391 ± 79	583 ± 92	98 ± 27	
Experimental	4	235 ± 18	−10‡ ± 1	245§ ± 67	217‡ ± 23	54§ ± 11	

* Values shown are averages ± sample standard deviations.

† AdX, animals adrenalectomized 5 to 7 days prior to experiment and maintained with 1 per cent NaCl in their drinking water.

‡ $P < 0.1$ per cent.

¶ $P = 0.5$ per cent.

§ $P = 2.5$ per cent.

TABLE 3

TOXICITY OF ACTINOMYCIN D GIVEN INTRAVENOUSLY TO ADULT MONGREL DOGS*

Observed for 14 Days After the Final Injection

Dose (γ/kg./day)	No. success. daily doses	Mortality	Day of death
200	1	2/2	1, 3
100	1	4/4	5, 5, 5, 5
50	1	0/4	
20	10†	4/4	5, 7, 11, 11
15	10	2/2	7, 11
10	10	1/6	16‡

* Equal numbers of both sexes used for each dose tested.

† A maximum of 10 doses was given on successive days except week ends, that is, 10 doses in eleven to thirteen days.

‡ Killed *in extremis*.

Fifteen of the 22 dogs (TABLE 3) became febrile at some time; in 10 of these 15 fevers developed within the first four days of intoxication. FIGURE 5 gives different examples of these early fevers. The delayed nature of the response indicates that actinomycin D does not act as a classic pyrogen.[16,17]

Other prominent effects in two of the dogs listed in TABLE 3 are given in detail in FIGURE 6. This pair died on day 5 after a single dose of 100 γ/kg. Weight loss began after the second day coincident with loss of appetite. Bloody diarrhea appeared on the third. By the fourth day the hematocrits were elevated. Reticulocytopenia, lymphopenia, and eosinopenia were prominent; little change was seen in platelet counts and the neutrophils increased temporarily.

Another change is evident in FIGURE 6: both dogs were hypochloremic by the fourth day, an effect consistent with their dehydrated appearance. Alterations in extracellular electrolytes in serum are shown again in FIGURE 7, which describes a second pair of dogs given 100 γ/kg. As did the previous pair, these also died on day 5. The constancy of the serum potassium suggests that the electrolyte imbalance was not complicated by or mediated through adrenal cortical mechanisms.

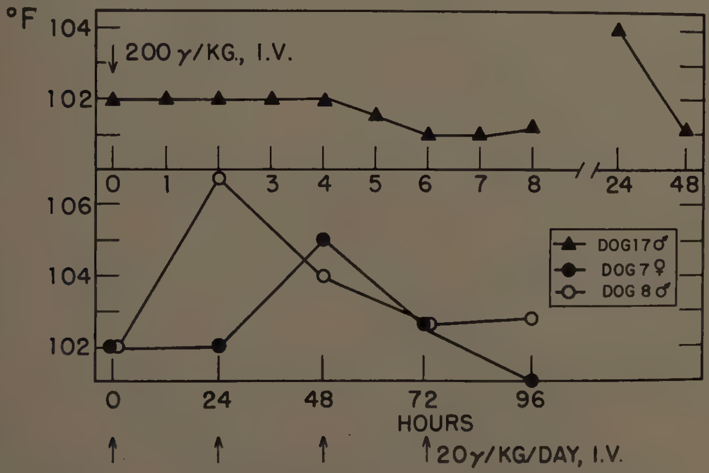

FIGURE 5. Rectal temperatures in dogs receiving actinomycin D.

Studies similar to those shown in FIGURE 6 were carried out with 12 additional dogs receiving other doses of actinomycin D (TABLE 3). Of a pair given 50 μg./kg., a nonlethal amount, one developed a transient granulocytosis and both had transient episodes of lymphopenia and reticulocytopenia during the first week. All four of the animals given daily doses of 20 γ/kg. had reticulocytopenia before death; one of the four showed a transient granulocytosis, one a lymphopenia, and two showed eosinopenia. The pair receiving 15 γ/kg./day showed lymphopenia and reticulocytopenia and a transient moderate neutropenia. Of the dogs given multiple doses of 10 γ/kg./day, two showed only transient reticulocytopenia and survived; the fate of a second

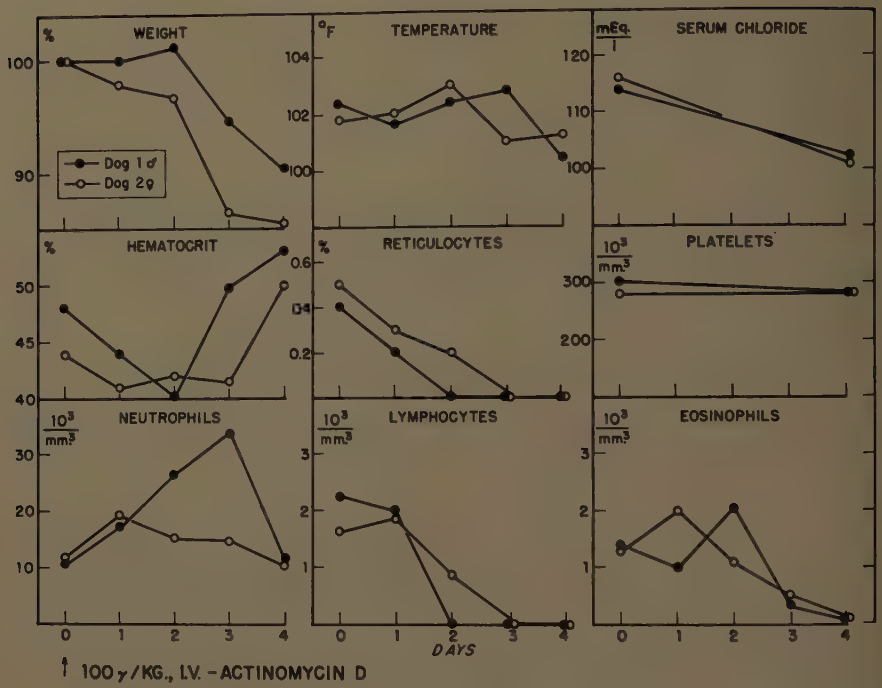

FIGURE 6. Effects of actinomycin D in dogs.

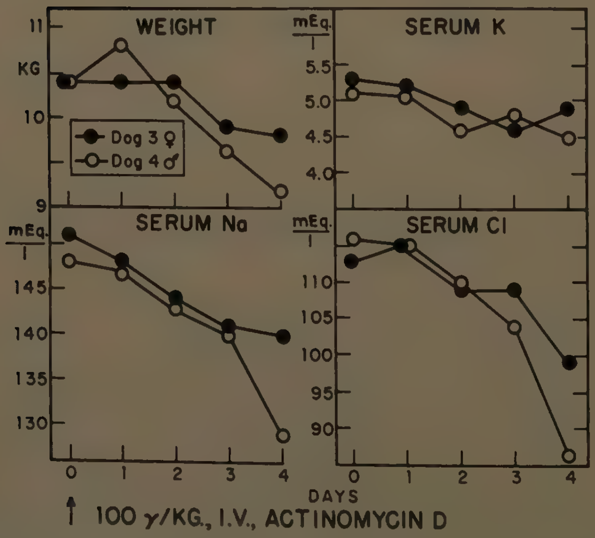

FIGURE 7. Changes in serum electrolytes in dogs receiving actinomycin D.

pair is given in FIGURE 8. One of these, dog 13, survived. It had a brief episode of mild fever at 13 days, reticulocytopenia, and a transient neutrophilic granulocytosis. The other animal, dog 14, was the only example in the series of a change typical of extensive bone marrow arrest. An absolute neutropenia, a reticulocytopenia, and a severe thrombocytopenia developed. The animal also had two febrile episodes, an earlier one between days 3 and 7 before the neutrophil counts were depressed, and a later one at days 15 and 16 coincident with neutropenia. Blood clotting times were significantly increased at day 14 coincident with the platelet depression. Severe hypochloremia was another notable alteration in this animal.

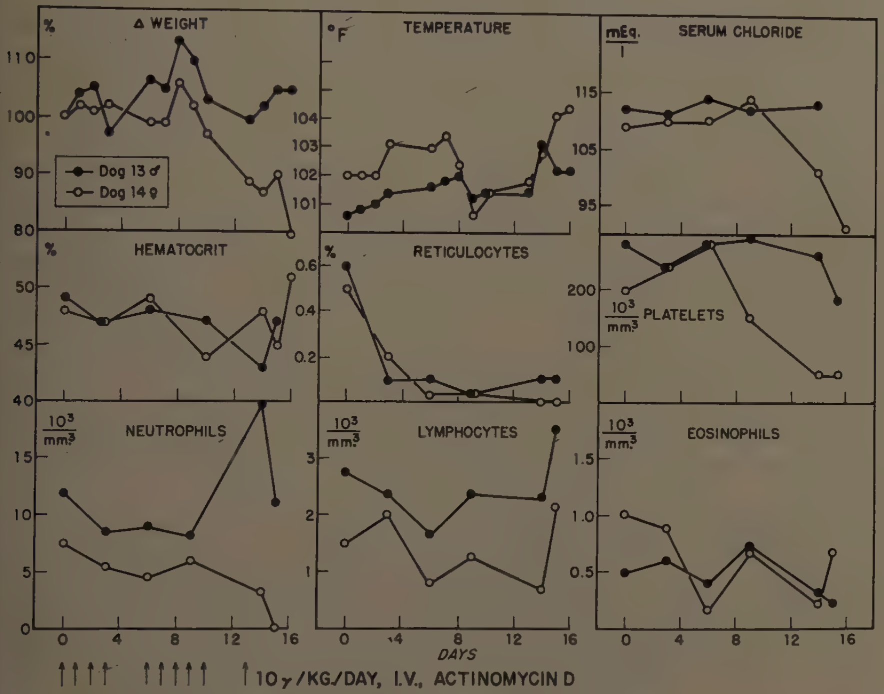

FIGURE 8. Effects of actinomycin D in dogs.

In addition to the measurements just presented, analyses were done in each of the 14 dogs for serum alkaline phosphatase, plasma prothrombin time, serum nonprotein nitrogen, and blood glucose, as well as for retention of bromsulfalein. None of these parameters was significantly altered. No jaundice developed.

Pathological effects. The tissues of five dogs were studied for pathological changes. One was dog 14 of FIGURE 8, the one animal of TABLE 3 that was killed *in extremis*. The others, not included in TABLE 3, were a pair of dogs given single doses of 100 γ/kg. and a pair given four daily doses of 20 γ/kg./day; all four were killed at day 4.

The gross autopsy of dog 14 revealed widespread hemorrhages (mostly petechial) in such tissues as the adrenal cortex, esophagus, mucosa of stomach and of small intestine, lung, and pericardium. The mesenteric lymph nodes were dark red. There were areas of atelectasis in different lobes of the lung and a small amount of froth in the trachea. Of the other four dogs, two had small areas of purple discoloration in the lungs; the tonsils of three were dark purple or grey-brown in color; the rectums of two had small hemorrhages; the intestinal contents were sparse, fluid and, in one, blood-stained.

Lesions were seen microscopically in each of the five dogs in bone marrow, intestines, and lymphoid tissues. There was a significant reduction in the quantity of hematopoietic cells in vertebral bone marrow of the two dogs given 20 γ/kg. In the other three the vertebral marrow was essentially aplastic; the nucleated cells consisted of a few neutrophils, lymphocytes, megakaryocytes, and isolated nests of reticulum cells. The marrow capillaries were congested and the stroma granular. The second severe change was in the mucosa of both small and large intestine. This consisted of swelling and atypia of the epithelial nuclei, glandular ectasia, clubbing of villi, moderate but significant atrophy, and congestion. Submucosal hemorrhages were also evident.

Lymphoid changes were widespread. The tonsils had microabcesses, hemorrhages, congestion, and infiltration with polymorphonuclear leukocytes. In dog 14 there was superficial ulceration with bacterial colonies. The lymph nodes, although normal in the dogs given 20 γ/kg., had reduced numbers of small lymphocytes and congested sinuses in the other three animals. The lymphoid follicles of spleen were reduced in size and the red pulp contained only erythrocytes. The thymi of dog 14 and the pair given 100 γ/kg. had numerous areas of karyorrhexis.

The hemorrhages mentioned above, in the gross autopsy of dog 14 were seen in the same sites by microscopic study and, in addition, in the thymus and perithymic tissues. Their widespread occurrence in this animal alone was probably related to the thrombocytopenia (FIGURE 8). The pulmonary lesions seen grossly in three of the dogs proved to be foci of early bronchopneumonia. In dog 14 these foci were atelectatic and contained bacterial colonies.

Except for the alterations just described, there were no conspicuous consistent lesions in any of the other thoracic or abdominal viscera.

Comments

The pathological effects of actinomycin D consist of local lesions in tissues directly exposed at sites of injection and of systemic lesions in the bone marrow, lymphoid tissues, and intestinal epithelium. Lymphocytotoxicity is, at least in part, a direct effect not mediated through stimulation of the pituitary-adrenal cortical system. In this respect actinomycin D closely resembles ionizing radiations and nitrogen mustards.[15] It also resembles these as well as most other antitumor agents in its selective cytotoxicity in bone marrow and intestinal epithelium. The triad of tissues that responds to the systemic effects of actinomycin is the same group of normal tissues in which a high rate of cell proliferation is a major functional differentiation in the adult mammal.[18]

The systemic lesions caused by actinomycin D explain certain of the overt signs of intoxication; for example, diarrhea, and the dehydrated appearance of intoxicated animals are reasonably related to the presence of severe alterations in the intestinal mucosa. However, the survival time after administration of minimal lethal doses is considerably shorter than that of animals treated with similar doses of other antitumor agents that cause equally severe selective damage to intestine and hematopoietic tissues.[19-21] The shorter survival with actinomycin D may account for the observation that animals killed a day or two before the expected time of death can have extremely hypoplastic bone marrows and at the same time still show relatively high numbers of granulocytes and thrombocytes in the blood. Presumably there is insufficient time for changes in peripheral blood to reflect the full consequence of hematopoietic arrest. Hackmann has previously stated that the more acute disturbances caused by the actinomycins differentiate them from the other cytostatic antitumor agents.[4,5] In addition, he has called attention to a possible similarity between these acute actions and the effects of bacterial endotoxins. The present studies have also revealed endotoxinlike properties,[16,17] for example, the induction of granulocytosis in rats and dogs and of fevers in dogs, although, as mentioned above, the febrile responses of dogs were more delayed than those that are typical reactions to bacterial pyrogens. While the endotoxinlike effects of the actinomycins may be nothing more than coincidental mimicry, other possibilities should not be overlooked. It is conceivable that early damage in the intestinal mucosa permits penetration of enteric toxins into the circulation. Such a mechanism would be compatible with the delayed nature of the pyrogenic responses. It is also conceivable that the agent, through its cytotoxic effects, promotes an early release of pyrogenic polysaccharides from normal cells. Such moieties with properties akin to the polysaccharides of bacterial endotoxins have recently been demonstrated in a wide spectrum of normal mammalian tissues.[22] It may prove profitable to consider these possibilities in future studies of the mode of action of the actinomycins.

Acknowledgments

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THE EFFECT OF ACTINOMYCINS ON EXPERIMENTAL TUMORS

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The antitumor effect of actinomycins is the only rationale for their therapeutic use in humans. It was observed relatively late in animal experiments—only several years after Waksman and Woodruff (1940) had isolated and described the first actinomycin. While the effects of bacterial toxins and metabolites of bacterial cultures on animal and human tumors were long known and therapeutically utilized, the antibiotic substances have played a role in the treatment of cancer for only seven or eight years. To the best of my knowledge, actinomycin C, first isolated by Brockmann *et al.* in 1949, was—under the trade name Sanamycin—the first antibiotic and is still the only actinomycin generally available for the treatment of malignant disease.

In recent years—to be precise, only since Waksman (1940), at the Sixth International Congress on Microbiology in Rome, Italy, September 7, 1953, referred to our experiments and to the clinical studies carried out by Schulte—more than 250 scientific, chemical, and biological papers have dealt with these natural substances, which are of very great interest in cancer research. Various reasons, especially the inherent properties of actinomycins and today's great chemico-technical possibilities for producing antibiotics, account for this interest. A prominent characteristic of the actinomycin class is its ability to produce many variations of its substances. Major points both of the phenomenon of actinomycin variations and of the differing properties of the various products were explained by the elucidation of the structure of actinomycins by Brockmann and his co-workers, by the results of paper-chromatographic studies, and by the success in producing actinomycins biosynthetically.

Table 1, exhibiting the toxicity of several actinomycins, shows the great differences in this respect.

The occurrence of actinomycin variations, of course, widens the prospects of finding other substances in the actinomycin class that may have interesting and even better properties, and thus of achieving certain advances in treatment. Animal experiments have shown that actinomycins have a rather complex mechanism of action. Studies of their effect on the animal body have indicated several components of action, which may vary with the actinomycins according to the amino acid composition of their peptide chains.

The bacteriostatic effect, utilized in the isolation of the substances, is probably of no significance for their use in cancer therapy. In experiments on mice the most obvious effect of actinomycins A, C, and D, and of some others is the inhibition of the spleen, which Robinson and Waksman described in their toxicological studies in 1942. In our experiments actinomycin C showed a strongly inhibitory effect not only on the spleen but also on the thymus and lymph nodes. The activity is directed chiefly, and in a higher degree than is the case with most cytostatic substances, against cells and tissues of the lymphatic system. The experimental and clinical effects therefore bear a certain resemblance to those of X rays.

In this connection may I also point out the interesting reactions that Tan (1958) observed on irradiated skin areas during the administration of actinomycin D. The actinomycin effect does not, however, fully resemble the biological effect of X-ray irradiation. In particular the effect on the centers of formation of white and red blood cells and the reproductive glands is considerably less with actinomycins than with irradiation and other so-called radiomimetic agents.

The various actinomycins do not possess the same degree of lymphotropic potency, and this effect is more marked in mice than in rats. Clinically, the lymphotropic component of action is utilized in the treatment of Hodgkin's disease, in which the therapeutic value of actinomycin C was first demonstrated by Schulte (1952).

A further characteristic, especially noticeable in animals treated with actinomycins, is the hemorrhagic effect. High doses in animals give rise to hemorrhages in various tissues and hemorrhagic inflammation of the intestinal mucosa associated with blood-stained diarrhea. A number of microbiological metabolites, in particular, the bacterial toxins—for example, of *Bacillus prodigiosus* and several microorganisms causing enteritis—have long been known to elicit hemorrhagic reactions in tissues. It may be that the newly formed vessels in tumor tissue are especially sensitive to capillary poisons. A capillary-active component also is inferred from the pattern of action of several substances other than the bacterial toxins of *B. prodigiosus* and other microorganisms that have long been used in tumor treatment: for example, arsenic, which is still employed in Hodgkin's disease and was previously tried in treating other tumors. It is therefore interesting and perhaps not insignificant, with regard to the therapeutic effect of actinomycins, that certain doses of these natural substances also may cause capillary hemorrhage.

TABLE 1

TOXICITY OF SEVERAL ACTINOMYCINS*

Actinomycin	LD_{50} mg./kg. mice I.V.
C	1.0
D	0.7
E_1	1.0
E_2	1.0
F_1	8.0–10.0
F_2	2.0
F_3	5.0
F_4	5.0

* C. Hackmann. Krebsforschung und Krebsbekämpfung Bd. III, Seite 334, Sonderband zur Strahlentherapie Bd. 41, 1959. Reprinted by permission of Strahlentherapie.

The decisive factor in the antitumor action of actinomycins is, however, their effect on the cells of tumor tissue and on the process of cell division. Numerous authors have observed a therapeutic effect with almost all actinomycins on great numbers of experimental animal tumors. TABLES 2 to 4 list the actinomycin investigations of the past decade (see also *References*).

Concerning the tumor-inhibiting action of the actinomycins, of greatest

TABLE 2
ACTINOMYCIN C

Tumor	Species	Authors
Ehrlich carcinoma solid	Mouse	Hackmann (1952)
Sarcoma 37	Mouse	Hackmann (1952)
Walker carcinoma	Rat	Hackmann (1952)
3RC Carcinoma	Mouse	Field *et al.* (1955)
Crocker carcinoma	Mouse	Field *et al.* (1955)
Leukemia L 4946	Mouse	Field *et al.* (1955)
Adenocarcinoma C3HBA	Mouse	Pugh *et al.* (1956)
Gardner lymphosarcoma	Mouse	Pugh *et al.* (1956)
Jensen sarcoma	Rat	Hackmann and Schmidt-Kastner (1956)
Rous sarcoma	Chicken	Hackmann and Schmidt-Kastner (1956)
Lymphosarcoma	Mouse	Borić *et al.* (1957)
Sarcoma AK	Mouse	Borić *et al.* (1957)
Yoshida sarcoma	Rat	Borić *et al.* (1957)
Crocker sarcoma 180	Mouse	Hsü Bin *et al.* (1959)

TABLE 3
ACTINOMYCIN D

Tumor	Species	Authors
Mammary adenocarcinoma	Mouse	S. Farber (1955)
Melanoma S91	Mouse	S. Farber (1955)
Myeloid leukemia	Mouse	S. Farber (1955)
Lymphoid leukemia	Mouse	S. Farber (1955)
Mammary adenocarcinoma C3HBA	Mouse	L. H. Pugh *et al.* (1956)
Lymphosarcoma(?)	Mouse	L. H. Pugh *et al.* (1956)
Sarcoma 180	Mouse	F. J. Gregory *et al.* (1956)
Gardner lymphosarcoma	Mouse	F. J. Gregory *et al.* (1956)
Ehrlich carcinoma solid	Mouse	K. Sugiura (1956)
Ehrlich carcinoma ascites	Mouse	K. Sugiura (1956)
Krebs 2 ascites carcinoma	Mouse	K. Sugiura (1956)
Sarcoma 180 ascites	Mouse	K. Sugiura (1956)
Ridgeway osteogenic sarcoma	Mouse	K. Sugiura and M. Schmid (1956)
Jensen sarcoma	Rat	Ch. Hackmann and G. Schmidt-Kastner (1956)
Rous sarcoma	Chicken	Ch. Hackmann and G. Schmidt-Kastner (1956)
Human sarcoma 1	Rat	Ph. Merker *et al.* (1957)
Human epithelioma 3	Rat	Ph. Merker *et al.* (1957)
Adenocarcinoma C-55	Mouse	J. A. DiPaolo *et al.* (1957)
Mammary carcinoma C3HBA	Mouse	J. A. DiPaolo *et al.* (1957)
Krebs 2 ascites	Mouse	J. A. DiPaolo *et al.* (1957)
Ehrlich carcinoma ascites	Mouse	K. Sugiura *et al.* (1958)
Adenocarcinoma E0771	Mouse	K. Sugiura *et al.* (1958)
Carcinoma 1025	Mouse	K. Sugiura *et al.* (1958)
Harding-Passey melanoma	Mouse	K. Sugiura *et al.* (1958)
Sarcoma 180 ascites	Mouse	K. Sugiura *et al.* (1958)

importance is their direct effect on the cells of tumor tissue and, especially, on the process of cell division. This effect has been extensively investigated. My associates and I, using actinomycin C in studies of the duodenum of the mouse, mouse sarcoma 37, and Walker carcinoma of the rat, observed an effect on the quiescent cells and the early stages of cell division. Further research with actinomycin M was carried out by the Italian workers Monesi and Veronesi (1955) and Craveri and Veronesi (1957). These workers found that even very great dilutions cause in proliferating cells of chick embryo tissues a decrease in the rate of mitosis, retardation of the initial phases of mitosis, cytoplasmatic and nuclear vacuolation, nuclear hypertrophy, pycnosis, karyorrhexis, and karyolysis, while they saw little effect in the nonproliferating cells. The germinative tissue of the rabbit testis was exceptional in that it was unaffected. Furthermore, note was made of chromosome lesions, adhesions, breakages, and bridges. The investigators came to the conclusion that actinomycin C acts on the chromosomes chiefly before the prophase and that in its effect it resembles X rays.

TABLE 4

Actinomycin	Tumor	Species	Authors
A	Ehrlich carcinoma ascites	Mouse	Nitta *et al.* (1955)
A I.V.	Gardner lymphosarcoma	Mouse	Pugh *et al.* (1956)
D I.V.	Adenocarcinoma C3HBA	Mouse	Pugh *et al.* (1956)
4A-2	Ehrlich carcinoma ascites	Mouse	Nishibori (1956)
4A-2	Ehrlich carcinoma solid	Mouse	Nishibori (1956)
4A-2	Yoshida sarcoma	Rat	Nishibori (1956)
E_1	Jensen sarcoma	Rat	Hackmann and Schmidt-Kastner (1956)
E_1	Walker carcinoma	Rat	Hackmann and Schmidt-Kastner (1956)
E_1	Rous sarcoma	Chicken	Hackmann and Schmidt-Kastner (1956)
F_0, F_1, F_2,	Walker carcinoma	Rat	Hackmann and Schmidt-Kastner (1956)
F_3, F_4, F_5	Rous sarcoma	Chicken	Hackmann and Schmidt-Kastner (1956)
J	Ehrlich carcinoma ascites	Mouse	Nishibori (1956) Oota *et al.* (1957)
J	Ehrlich carcinoma ascites	Mouse	Sugiura *et al.* (1958)
J	Krebs 2 ascites	Mouse	Sugiura *et al.* (1958)
J	Sarcoma 180	Mouse	Sugiura *et al.* (1958)
J	Carcinoma 1025	Mouse	Sugiura *et al.* (1958)
J	Ridgway osteogenic sarcoma	Mouse	Sugiura *et al.* (1958)
J	Crabb sarcoma	Hamster	Sugiura *et al.* (1958)
J	Yoshida sarcoma ascites	Rat	Oota *et al.* (1957)

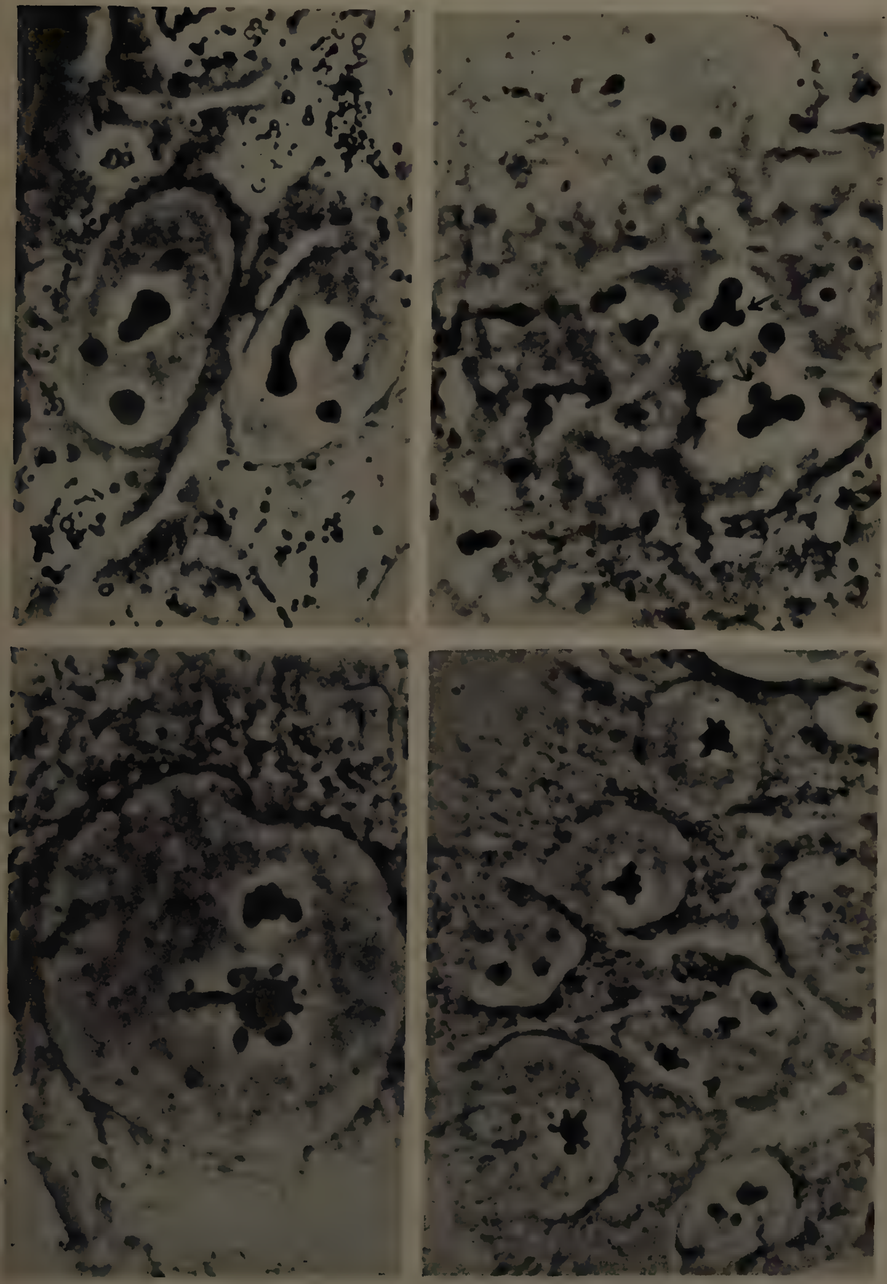

FIGURE 1. *Top left*, control HeLa cells. *Bottom left*, HeLa cells with actinomycin C (concentration in solution, 10^{-6}) after 5 hours; high magnification. *Top right*, HeLa cells with actinomycin C (concentration in solution, 10^{-7}) after 5 hours. *Bottom right*, HeLa cells with actinomycin C (concentration in solution, 10^{-6}) after 5 hours.

R. Bierling (unpublished communication) examined by phase microscopy in our laboratory the effect of actinomycins C, D, F_1, and F_3 on live tissue cultures of the human cervix carcinoma HeLa and on connective tissue cells of the human ovary. The test substances were used in concentrations of 10^{-5} to 10^{-10} in Tyrode's solution at *p*H 7.8 and an isotonic value of 0.58°. The cytostatic effect became manifest *in vitro* within 24 hours; it consisted in destruction of the tissue at concentrations as low as 10^{-6} and in disturbances of cell division at concentrations as low as 10^{-8}. All the actinomycins in Bierling's experiments effected in as little as two to five hours characteristic changes in the structure of the nucleoli in all types of cells studied. It has not been possible, however, to differentiate the characteristic properties of the actinomycins from the morphologic changes observed. The changes are quantitatively graded in the various ranges of concentrations. They extend from almost complete elimination of the nucleoli at a concentration of 10^{-5} through changes in vesicular structure at concentrations of 10^{-6} to 10^{-7} to marked condensation of the nuclear substance and, eventually, almost normal nucleoli at still lower concentrations, when the nucleoli show characteristic vesicles at their margins.

These changes precede cytolysis and are also observed in concentrations showing no other cytostatic effect. Feulgen's staining of the cultures demonstrates the decrease in, or absence of, desoxyribonucleic acid in those zones of the nucleolonema that show changes by phase microscopy. A remarkable observation in Bierling's experiments (unpublished) was that the mitochondria in the treated cells remained unaffected by degenerative changes during the whole period of the test, and the lipoid drop in the cytoplasm, which occurs under the action of other cytostatic substances—for example, alkylating agents —did not form.

The described morphologic changes due to the action of actinomycins differ clearly from the effects of other cytostatic substances; they appear to be characteristic of actinomycins and have been observed in dilutions of above 10^{-7} only with these natural substances. According to the results of these studies, actinomycins have some properties by which they differ from other agents used in tumor treatment and account for the interest attached to their therapeutic use. The possibility of variations in the microbiological field and the ability of microorganisms to form even highly complex molecules open vast prospects for research. The finding of further microbiological agents with antitumor effects proves that development in this field is not yet complete. The actinomycins have a special mode of action and special properties: they do not affect the bone marrow and their effect on cells may be experimentally demonstrated even in very great dilutions. They are, therefore, particularly suited to long-term administration in doses adapted to individual tolerance.

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THE EFFECT OF ACTINOMYCIN D ON A SPECTRUM OF TUMORS*

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Thirty years ago Fleming[1] isolated from a mold, *Penicillium notatum*, a substance with antimicrobial activity. Since then many antibiotics have been isolated from culture filtrates of soil bacteria and actinomycetales. According to Selman A. Waksman (personal communication), there are now about 500 known antibiotics. Of these only about 30 are in clinical use and still fewer, approximately 1 per cent, show antitumor activity in humans.

Experimental evidence of the last few years indicates that certain antibiotics have strong antitumor effects on certain animal neoplasms.[2-4] Such studies continue to yield new information that may be applicable to trials against human cancer. This report consists of observations on the effect of actinomycins C, D, and J on the growth of 16 mouse tumors, 6 rat tumors, 3 hamster tumors, 3 ascites tumors, 1 mouse leukemia, 1 mouse virus leukemia, and 1 chicken tumor.

Materials and Methods

History, biological properties, and cytological descriptions of most of the tumors that my associates and I have used have been presented elsewhere.[5-7]

The methods employed in the chemotherapy studies to be described were as follows:

Solid tumors:[5] Subcutaneous implantations of tumor fragments into healthy young animals (18- to 22-gm. mice and 80- to 100-gm. rats, hamsters, and chicks) were made by the usual trocar method, a single implant being placed in the axillary region of an animal and in the wing fold of a bird. In every set of experiments, tumor-bearing animals were divided into two groups, one for control and the other for treatment with antibiotics. The progress of the tumors in the animals was recorded graphically from caliper measurements of the tumors in two diameters made at weekly intervals for 4 weeks after the transplantation.

Ascites tumors:[8] Intraperitoneal injection of 0.1 ml. of the fluid, containing about 1 million cancer cells, was made into each mouse in the inguinal region. Mice so inoculated regularly develop large amounts of a milky or hemorrhagic ascites in 7 to 14 days and die in 10 to 16 days.

Friend virus leukemia:[9] Intraperitoneal injection of 0.2 ml. of a 10 per cent saline homogenate of leukemic spleens was made into each mouse in the inguinal region. This transplanted leukemia killed the mice, with marked enlargement of liver and spleen, in 2 to 4 months. The effects of the antibiotics have been evaluated through comparison of the weights of spleens in treated and untreated

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infected mice 3 weeks after the intraperitoneal injection of the leukemic spleen homogenate. At this time the spleens of the leukemic mice generally weighed about 2 gm. For convenience in routine screening, the 3 weeks' observation period instead of the survival time was chosen.

Intraperitoneal injection of the antibiotics at or near maximal toleration doses was begun 24 hours after the inoculation with tumor material and was continued once daily for 7 days. When, during this procedure, a substance showed a marked inhibitory or destructive effect, it was tested on well-established, 7-day-old tumors.

Concentrated solutions of actinomycins C, D, and J in ethanol were diluted in saline. The injection volumes of the solutions were 0.5 cc. once daily.

The degree of inhibition of the growth of solid tumors was graded according to the following scheme: −, no effect (tumor growth of three quarters or more of the diameters of control tumors); ±, slight inhibition (tumor growth of one half to three quarters of the diameters of control tumors); +, moderate inhibition (tumor growth of one fourth to one half of the diameters of control tumors); ++, marked inhibition (no growth, or growth of approximately one fourth of the average diameter of control tumors); and +++, complete inhibition or destruction of tumors.

The degree of inhibition of ascites tumor growth was graded according to the following scheme: −, no effect (marked abdominal distention, fluid volume increased to three fourths or more of control tumors, 10- 25-cc. ascites); ±, slight inhibition (moderate abdominal distention, fluid volume increased to about one half of control tumors, 5- 9-cc. ascites); +, moderate inhibition (slight abdominal distention, fluid volume increased to about one fourth of control tumors, 1- 4-cc. ascites); ++, marked inhibition (no abdominal distention, no gross ascites); and +++, complete inhibition or destruction of ascites.

The degree of inhibition of virus leukemia was graded according to the following scheme: −, no effect (no change in leukemic spleen weight); ±, slight inhibition (about 25 per cent spleen-weight inhibition); +, moderate inhibition (about 50 per cent spleen-weight inhibition); ++, marked inhibition (about 75 per cent spleen-weight inhibition); and +++, complete inhibition (no difference in spleen weight from that of normal nonleukemic animals).

Results

The results obtained with antibiotics tested at or near maximal toleration doses are summarized below and in TABLE 1. Test groups consisted of 5 or 10 animals each, and each experiment was repeated; the data presented are averages of the results of multiple experiments. The evaluation of effects has been based on observations made 2 weeks after tumor inoculation, except in the case of Friend virus leukemia: because of the long survival time of these leukemic mice, observations were made after 3 weeks.

From the data in TABLE 1 it is evident that at well-tolerated doses actinomycin D had almost no inhibitory effect on the majority of solid tumors of mice, rats, hamsters, and chickens, except for a destructive effect on Ridgway osteogenic sarcoma, a marked effect on carcinoma 1025, and moderate effects on mammary adenocarcinoma E0771 and Harding-Passey melanoma. Repeated intraperitoneal injections of 0.025 mg./kg. of actinomycin D had a marked inhibitory effect on nonpigmented Harding-Passey melanoma. The antibiotic had a destructive effect on all three ascites tumors, but almost no inhibitory effect on the respective solid tumors. Intraperitoneal injections of actinomycin D into mice 5 to 7 days after injection with ascites tumors were less effective than those begun 1 day after injection (complete regression of about 50 per cent of ascites tumors rather than 100 per cent).

TABLE 1

EFFECT OF ACTINOMYCINS ON MOUSE, RAT, HAMSTER, AND CHICKEN TUMORS

Tumor	Actinomycin (0.025 mg./kg./day)*		
	D	C	J
Ridgway osteogenic sarcoma	+++	+++	++
Sarcoma 180 (ascitic)	+++	+++	+++
Ehrlich carcinoma (ascitic)	+++	+++	+++
Krebs 2 carcinoma (ascitic)	+++		+++
Carcinoma 1025	++	++	++
Adenocarcinoma E0771	+	−	±
Harding-Passey melanoma	+		
Sarcoma 180 (solid)	±	±	±
Jensen sarcoma	±	±	+
Fortner small intestine tumor No. 1	±	−	±
Sarcoma T241	−	−	±
Sarcoma MA387	−		−
Ehrlich carcinoma (solid)	−	−	±
Bashford carcinoma 63	−	−	−
Miyono adenocarcinoma	−	−	+
Lewis bladder carcinoma	−		−
Lewis lung carcinoma	−	−	±
Wagner osteogenic sarcoma	−	++	±
Mecca lymphosarcoma	−	−	−
Gardner lymphosarcoma	−		−
Glioma 26	−	−	−
Friend virus leukemia	−	+	+
Leukemia L 4946	−	−	−
Flexner-Jobling carcinoma	−	−	±
Walker carcinosarcoma 256	−	−	±
Murphy-Sturm lymphosarcoma	−	−	±
Iglesias ovarian tumor	−	−	−
Iglesias adrenal tumor	−	−	−
Crabb hamster sarcoma	−	++	++
Fortner pancreatic tumor No. 1	−	−	−
Rous chicken sarcoma	−	−	−

* Symbols: −, no effect; ±, slight inhibition; +, moderate inhibition; ++, marked inhibition; +++, complete inhibition or destruction of tumors.

Treating 7- to 10-day-old Ridgway osteogenic sarcomas, having diameters of 5 to 10 mm., with 0.025 to 0.05 mg./kg./day of actinomycin D for 7 days caused growth stoppage for a few days and then complete regression of the tumors (96 out of 120 tumors, or 80 per cent cure) in 2 to 3 weeks. There was no reappearance of tumors during experimental periods of 2 to 3 months, and at necropsy no neoplastic material was found at the former site of the tumor. Well-established carcinoma 1025 responded well to the antitumor activity of actinomycin

D; with 0.05 mg./kg. of actinomycin D the growth of 29 seven-day-old tumors was much inhibited, but there was no complete tumor regression.

Because actinomycin D had a definitely inhibitory effect on the growth of transplantable mammary carcinomas, it was tested on 30 Swiss albino mice with recently developed, spontaneous mammary adenocarcinomas. In various groups of these mice intraperitoneal injections of the antibiotic (0.025 to 0.05 mg./kg./day for 7 to 14 days) had almost no inhibitory effect on these spontaneous mammary carcinomas.

Actinomycin D also was used in the treatment of 25 Swiss albino mice with small methylcholanthrene-induced sarcomas. The tumor-bearing animals were injected intraperitoneally with 0.025 mg./kg. of actinomycin D once daily for 14 days. The results showed that this antibiotic had no inhibitory effect upon chemically induced sarcomas in mice.

Experiments with actinomycin D[12] (Selman A. Waksman, Rutgers, The State University, New Brunswick, N. J., and Merck Laboratories, Rahway, N. J.) have been repeated with crystalline actinomycin C[13] (Farbenfabriken Bayer A. G., West German Federal Republic) and actinomycin J (M. Ooka, Nippon Seibutsu Kagaku Keukyusho, Tokyo, Japan, personal communication). The results obtained with the present spectrum of tumors are summarized in TABLE 1. The carcinolytic action of actinomycins C and J was essentially the same as that of actinomycin D, except that C showed a markedly inhibitory effect on Wagner osteogenic sarcoma, whereas D and J showed almost none; both actinomycins C and J had a moderately inhibitory effect on Friend virus leukemia and a markedly inhibitory effect on Crabb hamster sarcoma, but actinomycin D had no inhibitory effect on either. Whether these differences have any significance in respect to the structural differences between actinomycins C, D, and J cannot be stated definitely at present. It is interesting to note that these actinomycins had a beneficial effect in Wilms' tumor, neuroblastoma, rhabdomyosarcoma, lymphosarcoma, Ewing's tumor, and melanoma.[10-12]

Summary

The cures of some transplantable animal tumors by actinomycin D are noted. These cures in certain animal tumors and the temporary effectiveness against some human neoplasms possibly offer hope for an eventual cure in some types of human cancer.

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COMPARATIVE EFFECTS OF ACTINOMYCINS II, III, AND IV ON SEVERAL ASCITIC TUMORS OF MICE*

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Interest in the actinomycins continues because they are selectively cytostatic against neoplasms in human as well as animal hosts. The antitumor effects in experimental animals of actinomycins, particularly those designated C and D, have been studied in the past decade by many investigators through out the world.[1-6] The results with certain animal-tumor test systems, such as the Ridgway osteogenic sarcoma, are dramatic.[7] In contrast, only slightly inhibitory effects on the subcutaneous sarcoma 180 tumor are observed.[6]

Clinical studies indicate that actinomycin D is the only chemotherapeutic agent active against Wilms' tumor and rhabdomyosarcoma.[8-10] Tan *et al.*[10] cite a case of Wilms' tumor in which the effect has lasted for 24 months following a single course of intravenous administration of the antibiotic, supplemented by several months of its oral administration. In a series of 49 cases, including various neoplasms treated only with actinomycin, 16 showed an objective improvement lasting from 1 to 10 weeks. The response of patients with Hodgkin's disease is variable;[11,12] temporary regressions following therapy with actinomycin C (Sanamycin) were described by several clinicians.[13,14] Actinomycin D is not of real value in the therapy of advanced neoplastic disease, as exemplified by breast cancer and adenocarcinoma. Moore *et al.*[15] reported that in some cases an enhanced tumor growth was observed. Despite the limited usefulness of this antibiotic at present, it is still of interest. Farber[8] has stated, "Actinomycin D . . . is one of the most powerful anticancer agents, by weight, so far discovered."

Natural actinomycins, originally given alphabetical designations (for example, actinomycin A, C, and D) are known to consist of mixtures of actinomycin components;[16,17] the individual components have been denoted by the addition of an Arabic or Roman subscript to the alphabetical designation. Since it now has been determined that many of these components are identical, the nomenclature proposed by Waksman *et al.*[18] will be used in this paper. For example, A_{II} and B_{II} are now designated actinomycin II. Similarly, A_{III} and B_{III} are known as actinomycin III. Actinomycin D is almost pure actinomycin IV, containing only trace amounts of actinomycins I, II, III, and V; actinomycin IV previously was identified as A_{IV}, B_{IV}, D_{IV}, C_1, I_1, and X_1.

In a study of the biosynthesis of actinomycins formed by *Streptomyces chrysomallus*, which under usual conditions yields actinomycin C, Schmidt-Kastner[19] produced two new series of actinomycins, designated E and F. The E series was obtained after the addition of DL-isoleucine to the basal medium; the F series was obtained after the addition of sarcosine. Katz and Goss[20] demon-

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strated that the addition of sarcosine to a chemically defined medium composed of D-galactose, L-glutamic acid, and several inorganic salts changes the relative percentage of actinomycins in the complex produced by *S. antibioticus.* Comparatively large amounts of actinomycins II and III now can be produced over the limited quantities that previously were available. These usually are not produced by *S. chrysomallus.* However, from the published data[21-23] it appears that actinomycins III and F_0 may be identical.

Preliminary data on toxicity of actinomycins II and III administered intraperitoneally have been reported from this laboratory.[24-26] The LD_{50} of actinomycin II was 6 mg./kg.; of actinomycin III, 1.5 mg./kg.; and of actinomycin IV, 1 mg./kg. Actinomycins II and III were found to possess an antitumor activity equal to, or better than, that of IV when 50 μg./kg. were administered intraperitoneally for 3 days to mice bearing the ascitic form of the Gardner lymphosarcoma. Such results indicate that actinomycins II and III merit further investigation; the therapeutic indices of II and III might be higher than that of actinomycin IV. Furthermore, the maximal therapeutic effect achieved with II or III might be better than that achieved with IV.

Materials and Methods

Four ascitic tumors of mice—an adenocarcinoma, Ehrlich Lettré hyperdiploid carcinoma, Gardner lymphosarcoma, and sarcoma 180—were employed in the studies. Unless otherwise noted, 10 male mice weighing about 20 gm. each were used for each experimental group. Two control groups, one treated with the highest concentration of solvent employed and one untreated, were included in the protocol. As a guide to toxicity, groups of 5 normal mice were treated with the highest concentration of actinomycin tested. The usual criterion of antitumor activity was an increase in the median survival time of treated mice over that of the solvent-treated control mice.

Pertinent information regarding the source of tumors, host strain of mice, and standard cell inoculum is presented in TABLE 1. The ascitic fluid was withdrawn aseptically from donor mice 7 days after implantation (10 days in the case of the Ehrlich Lettré hyperdiploid tumor). The diluent described by T. S. Hauschka (personal communication) was used for preparing suspensions of standard cell inoculums of ascitic fluid. This isotonic medium consisted of a solution of sodium chloride, calcium chloride, and potassium chloride, combined with Sorensen's buffer at *p*H 7. All suspensions were kept cold during manipulations. The mice were then placed randomly into experimental groups.

Three daily doses of actinomycin, beginning 24 hours after implantation of tumor, were given intraperitoneally, intravenously, or subcutaneously. Three or four concentrations of the actinomycins were employed in each test. Weights of individual mice were recorded at the beginning of the test period and at subsequent intervals. Dead mice were removed for necropsy daily. In about one half of the experiments (1500 mice), heart, lung, liver, spleen, kidney, and macroscopic tumor masses were examined histologically for efficacy and toxicity. The experiments were terminated 50 to 60 days after the beginning of the test.

Actinomycins II, III, and IV are produced by *S. antibioticus.* The crystalline actinomycins* were dissolved in propylene glycol (solubility, about 4 mg./ml.). It usually was necessary to heat the mixture in a water bath at 56° C. for 10 to 30 min., to ensure solution. The actinomycin solutions were diluted in sterile physiological saline so that the desired concentration could be injected in 0.5-ml. amounts. The final concentration of propylene glycol was not greater than 0.5 per cent.

Routine sterility tests were conducted on all tumor suspensions and antibiotics employed in these tests. The media used were thioglycollate broth and brain-heart infusion broth enriched with 0.5 per cent horse serum. Inoculated tubes were incubated for 1 day at 37° C. and for several days at room temperature.

TABLE 1
ASCITIC TUMORS EMPLOYED

Tumor	Source of tumor	Host mice	Source of mice	Standard cell inoculum/ mouse/day (0.5 ml.)
Adenocarcinoma S3A	George Klein, Karolinska Institutet, Stockholm, Sweden	A	Texas Inbred Mice Co., Houston, Texas	10×10^6
Ehrlich Lettré hyperdiploid carcinoma	Theodore Hauschka, Roswell Park Memorial Institute, Buffalo, N. Y.	Ha/ICR	Millerton Research Farm, Millerton, N. Y.	1×10^6
Gardner lymphosarcoma, 6C3-HED	Theodore Hauschka, Roswell Park Memorial Institute, Buffalo, N. Y.	C3H	Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.	4×10^6
Sarcoma 180	C. C. Stock, Sloan-Kettering Institute, New York, N. Y.	Ha/ICR	Millerton Research Farm, Millerton, N. Y.	2×10^6

Results

In the first series of experiments employing the 4 ascitic tumor systems, the various actinomycins were administered by the intraperitoneal route. On the basis of experience gained thus far, this test procedure has yielded reproducible results. The intravenous and subcutaneous routes were compared in later experiments, in view of the applicability of such data to the evaluation of therapeutic agents.

Intraperitoneal administration of actinomycins. The results of a representative trial, in which actinomycins II, III, and IV were administered for 3 successive days to mice bearing the ascitic Gardner lymphosarcoma tumor, are presented in TABLE 2. The presentation was designed to indicate the range of survival of the treated animals. The third column, deaths at 1 to 18 days after

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* Crystalline actinomycins II and III were prepared by Edward Katz of the Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J., and by Merck Sharp and Dohme Research Laboratories, Rahway, N. J. Crystalline actinomycin IV also was prepared at the Institute of Microbiology and originally was designated as actinomycin A_{IV}.[17]

TABLE 2

EFFECT OF INTRAPERITONEAL ADMINISTRATION OF ACTINOMYCINS II, III, AND IV ON ASCITIC GARDNER LYMPHOSARCOMA TUMOR IN MICE*

Actinomycin	Concentration/mouse/day for 3 days (μg.)	Mortality rate					Survivors at 52 days	Median survival (days)	Histopathol. exam., graded response	Therap. index†
		1 to 18 days	19 to 24 days	25 to 29 days	30 to 38 days	40 to 50 days				
II	2	●				●	●●●○○○○○	∞	2.0	125
	0.5	●		●	●●	●	○○○○○	50 (est.)	1.6	
	0.125	●●●●●●●		●●				15	2.8	
	0.03	●●●●●●●●●●						13	2.7	
III	2	T		●			●○○○○○○○	∞	0.8	30
	0.5	●	●		●●	○○	●●●○	50 (est.)	2.8	
	0.125	●●●●●●●●●	●					13	3.2	
	0.03	●●●●●●●		●●				13	3.2	
IV	2	T T ●	●●			●●	●○○	34	1.7	51
	0.5		●●●				●●●○○○○	∞	1.8	
	0.125	●●	●●●●			●○	○○	27	2.7	
	0.03	●●●●●●●●●						13	2.7	
Solvent-treated control		●●●●●●●●●●						14	3.5	
Saline-treated control		●●●●●●●●●●●●●●●●●●●					○	14	3.1	

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* Symbols: ●, ascites and/or solid tumor masses; ○, no macroscopic tumor; T, toxic death, third or fourth day.

† LD_{50}/ED, where the effective dose (ED) is the total amount of actinomycin required to give a 100 per cent increase in median survival time over median survival time of solvent-treated control mice.

implantation of tumor, includes early deaths presumably due to toxicity. Except in rare cases, all tumor-control animals died during this period. Those animals in which no macroscopic evidence of ascites or solid tumors was observed at necropsy are indicated in the table by open circles. The extent of tumor growth as determined by histological examination was graded on a 0 to 4 scale.

All three actinomycins showed significant activity against this tumor. When actinomycins II and III were administered at a dosage level of 2 μg. per mouse, 80 per cent of the mice survived the test period of 52 days. At necropsy many of these mice showed no macroscopic evidence of ascites or solid tumors. With actinomycin IV, a similar response was noted at the dosage level of 0.5 μg. per mouse. At the next highest concentration of actinomycin IV, 2 μg. per mouse, the median survival time was 34 days. Since there were 2 deaths from toxicity with this dosage, it appeared that toxicity was impairing the cytostatic effect. Such impairment has been observed in other experiments in which treatment with actinomycin IV was accompanied by deaths from toxicity. The graded response based on histological examination for tumor correlated well with the increase in survival time.

The therapeutic index of the actinomycins was calculated with the use of toxicity data obtained from the same lots of actinomycin as those used for therapy and also from the same host strain of mice (C3H). The effective dose chosen for these calculations was the one giving a 100 per cent increase in median survival time as compared with the median survival time observed in tumor-bearing mice injected with solvent alone. The therapeutic index of actinomycin II was twice that of actinomycin IV and four times that of actinomycin III.

In another experiment on the same tumor, designed to compare different lots of actinomycin II and employing actinomycin IV as a reference standard, results similar to those presented in TABLE 2 were obtained. The activity of actinomycin IV at levels of 2 μg. and 0.5 μg. was also similar to the values given in the table, but at the 0.125-μg. level it was higher.

A noninvasive ascitic adenocarcinoma tumor, S3A, was employed in two experiments. The results following the intraperitoneal administration of the actinomycins are presented in TABLE 3. The solvent-treated control animals died faster than the untreated controls; therefore it was necesasary to use the median survival time of the untreated tumor control animals as a basis of comparison. The greatest increase in survival time, 88 per cent, was obtained with actinomycin III administered at a level of 4 μg. per mouse. Increases in survival time with actinomycins II and IV were observed in a number of test treatment groups, but the activity did not follow the dose-response sequence. A possible reason for this lack of correlation between dosage and response in systems where high activity is not obtained is suggested in the discussion.

The results obtained with intraperitoneal administration of the three actinomycins to mice bearing the Ehrlich Lettré hyperdiploid tumor are presented in TABLE 4. Only one actinomycin was consistently active against this tumor system, and this activity was of a low order. However, the data contribute to the recognition of possible spectrum differences among the various actinomycins. Actinomycin IV was active at all three dosage levels, but actinomycin II gave an extension of survival time at only one level. A consistent dose response was not obtained. Actinomycin III was inactive at all levels tested.

If change in weight of the animals due to the volume of ascitic fluid produced is used as a criterion of activity, the slight differences between the actinomycins cannot be detected. For example, in a more severe test employing 20 million tumor cells per mouse instead of 1 million, only the average weight of the mice treated with actinomycin IV was significantly different from the average weight of the tumor-control animals (TABLE 5). In this test, all three actinomycins failed as judged by extension of median survival time.

TABLE 3

MEDIAN SURVIVAL TIME AFTER INTRAPERITONEAL ADMINISTRATION OF ACTINOMYCINS II, III, AND IV TO MICE IMPLANTED WITH ASCITIC ADENOCARCINOMA

Experiment no.	Treatment	Concentration/mouse/day for 3 days (μg.)	Median survival (days)
1	Actin. II	2 0.5 0.125	36 25 31
	Actin. IV	2 0.5 0.125	32 36 41
	Saline (control)		10
	Untreated (control)		28
2	Actin. III	4 2 0.5	47 23 19
	Actin. IV	4 2 0.5	7 (toxic) 30 41
	Solvent (control)		21
	Saline (control)		19
	Untreated (control)		25

Actinomycin D is known to possess some cytostatic activity against the ascitic form of sarcoma 180.[2] To broaden the ascitic tumor spectrum studies reported here, actinomycins II, III, and IV were tested against this tumor system. Actinomycin IV showed antitumor activity at the 4-μg. level, as evidenced by an extension of survival time of 100 per cent over that of solvent-treated control animals. Actinomycin II was without effect at dose levels of 0.5 μg. to 32 μg. per mouse. Actinomycin III showed no effect at levels of 0.13 μg. to 2 μg.

A comparison of the intraperitoneal doses effective against the four ascitic tumor systems is presented in TABLE 6. The figures for the Gardner lymphosarcoma tumor system were calculated from data obtained in two separate experiments in which identical curves were obtained. The effective doses for

TABLE 4

MEDIAN SURVIVAL TIME OF MICE IMPLANTED WITH EHRLICH LETTRÉ HYPERDIPLOID ASCITIC TUMOR FOLLOWING INTRAPERITONEAL ADMINISTRATION OF ACTINOMYCINS II, III, AND IV

Experiment no.	Treatment	Concentration/mouse/day for 3 days (μg.)	Median survival (days)	Mice with gross tumor (%)
1	Actin. II	32 8 2 0.5	22 19 30 19	90 90 70 100
	Actin. IV	4 2 0.5	35 36 32 (estim.)	100 80 60
	Solvent (control)		19	90
	Untreated (control)		18	100
2	Actin. III	4 2 0.5	20 19 22	90 100 100
	Actin. IV	0.5	36 (estim.)	80
	Solvent (control)		20	100
	Untreated (control)		21	100

TABLE 5

EFFECT OF INTRAPERITONEAL ADMINISTRATION OF ACTINOMYCINS II, III, AND IV ON BODY WEIGHT OF MICE IMPLANTED WITH EHRLICH LETTRÉ HYPERDIPLOID ASCITIC TUMOR

Treatment	Concentration/mouse/day for 3 days (μg.)	Tumor cells/mouse	Mice/group	Av. wt. 11 days after implant. (gm.)
Actin. II	2	20×10^6 None	10 5	40.6 31.4
Actin. III	2	20×10^6 None	10 5	41.5 30.1
Actin. IV	2	20×10^6 None	10 5	32.4 30.2
Solvent (control)		20×10^6	10	44.1
Untreated (control)		20×10^6	20	43.8
Normal (control)		None	5	31.6

the adenocarcinoma tumor represent two experiments in which either actinomycin II or III was compared with actinomycin IV. The increase in survival time observed after administration of 0.5 μg. actinomycin IV was 64 per cent; consequently, the curve was extrapolated to obtain the estimated effective dose. The data from two experiments were used in the calculations for the Ehrlich Lettré hyperdiploid tumor and the resultant doses were averaged. For sarcoma 180, the dose-response curves from one experiment were used for the calculation.

Intravenous administration of actinomycins. The relative efficacy of the actinomycins administered intravenously was tested against the Gardner lymphosarcoma and the ascitic adenocarcinoma. In the Gardner lymphosarcoma tests actinomycins II and IV were given to groups of 7 mice each. At the 2-μg. level actinomycin II showed no activity, although it had been active when administered intraperitoneally. Actinomycin IV was equally active by both test routes. The mice developed diarrhea during the course of this experiment perhaps obscuring valid results. Actinomycins II, III, and IV were inactive when administered intravenously to mice bearing the ascitic adenocarcinoma.

Table 6

Total Effective Dose* of Actinomycins II, III, and IV Producing 100 Per Cent Extension of Median Survival Time as Compared with Solvent-Treated Control Mice

Actinomycin	Ascitic tumor test system			
	Gardner lymphosarcoma	Adenocarcinoma†	Ehrlich Lettré hyperdiploid carcinoma	Sarcoma 180
II	0.05	Not effective near max. tolerated dose	Not effective at max. tolerated dose	Not effective at max. tolerated dose
III	0.05	0.64	Not effective at max. tolerated dose	Not effective at max. tolerated dose
IV	0.02	Estimated at 0.11	0.27	0.36

* Milligram per kilogram.

† Compared with untreated tumor-control mice. See text for explanation.

Subcutaneous administration of actinomycins. The subcutaneous route of administration was tested on three tumor systems. The results with one of them, the Gardner lymphosarcoma system, obtained after administration of actinomycins II and IV, are presented in TABLE 7. Some activity was noted when the antibiotics were administered subcutaneously, but its level was lower than that observed after intraperitoneal administration. With the ascitic adenocarcinoma no activity was observed when actinomycin III or IV was administered subcutaneously. At the 2-μg. level actinomycins II and IV showed no activity when administered subcutaneously to mice bearing the Ehrlich carcinoma.

Effect of higher concentrations of actinomycins. Attempts were made to determine the dose of actinomycin that produced the maximal effect. Complete cures (100 per cent survival at one dose level) were never obtained with the regimen employed in these tests, although relatively high concentrations were used. In the most sensitive of the four tumor test systems used, the Gardner lymphosarcoma, the best effect was obtained with actinomycin II administered intraperitoneally at a level of 2 μg. per mouse (TABLE 7). At this level, 1 of 10 mice died with tumor on day 46 of the experiment. The remaining 9 mice were killed on day 50 after tumor implantation, and there was no macroscopic evidence of ascites or solid tumor in any of them. At the highest dose level of actinomycin II administered, 8 μg., the survival rate was similar: 9 out of 10 on day 33 of the experiment. Of the 9 mice killed at the termination of the experiment, however, 2 showed tumors. The same type of results was obtained with actinomycin IV (TABLES 2 and 7), except that the 0.5-μg. level was superior to the 2-μg. level.

TABLE 7

COMPARISON OF EFFECTS OF INTRAPERITONEAL AND SUBCUTANEOUS ROUTES OF ADMINISTRATION OF ACTINOMYCINS II AND IV ON MEDIAN SURVIVAL TIME OF MICE IMPLANTED WITH ASCITIC GARDNER LYMPHOSARCOMA

Route	Treatment	Concentration/mouse/day for 3 days (μg.)	Median survival (days)	Mice with gross tumor (%)
I.P.	Actin. II	8	∞	30
		2	∞	10
	Actin. IV	2	50 (estim.)	60
		0.5	∞	40
	Solvent (control)		17	100
S.C.	Actin. II	8	∞	60
		2	17	90
	Actin. IV	4	∞	70
		2	50 (estim.)	100
	Solvent (control)		19	90
Untreated (control)			15	95

In the ascitic adenocarcinoma tumor system the same median survival time was obtained with 8 μg. actinomycin II administered intraperitoneally as was obtained in the untreated tumor control group. In the trials presented earlier in this report, actinomycin II was administered at doses up to 2 μg., and at 2 μg. a questionable degree of efficacy was observed. Raising the dose to 8 μg. did not increase the efficacy despite an apparent tolerance of this amount of agent.

When the Ehrlich carcinoma was used (TABLE 4), the maximal effect with actinomycin II was obtained at the 2-μg. level. A concentration of 8 μg. or even 32 μg. resulted in a shorter survival time as well as a higher percentage of mice with gross tumors. The maximal effect with actinomycin IV was obtained at the 0.5-μg. level. Although the 2-μg. or 4-μg. levels resulted in slightly longer survival times, higher percentages of animals with tumor were noted at these levels.

From these data it appears that the maximal effect of the actinomycins against the sensitive tumor is obtained at total levels that are one fifteenth to one fortieth that of the LD_{50} dose in the mouse tumor test systems evaluated here.

Discussion

Of the four ascitic tumor systems utilized in this study, the Gardner lymphosarcoma was the most sensitive to all three of the actinomycins studied. The reproducibility of results with this tumor system was excellent. The Ehrlich Lettré hyperdiploid carcinoma and sarcoma 180 tumors were not useful in determining differences between these closely related compounds. As expected, greater variation in response was encountered with the tumors grown in random-bred mice than with those grown in inbred mice.

The actinomycins showed only a low and sporadically retarding effect on the ascitic adenocarcinoma (S3A). Although this tumor was tested on an inbred host, a high degree of variation in response was observed. One difficulty associated with the use of the system was the wide difference in survival time between solvent-treated or saline-treated control animals and untreated control animals. Generally, the manipulations involved in the administration of nontoxic liquids to any tumor-bearing mouse produce a slight extension of survival time. Contrary to the generally observed effect, such manipulations of mice implanted with the ascitic adenocarcinoma produced a significant decrease in survival time. Furthermore, accurate cell counts of the ascitic fluid were difficult to make because the cells tended to clump. It would be desirable to test tumor response to implantation with a smaller number of cells than have been used in these studies. Within the limitations of the data, it appears that there were significant differences in the activity of the actinomycins against the ascitic adenocarcinoma. Actinomycin II was without effect at dose levels up to 8 μg. per mouse. Actinomycins III and IV exhibited a low order of activity, that is, less than 100 per cent increase in median survival time.

It has been stated previously[27] that the toxicity of various actinomycins parallels antimicrobial activity, but it was suggested also that antitumor activity does not necessarily correlate with any other known biological activity. To validate this suggestion, the testing of pure actinomycins against a variety of animal tumors would be essential. It would then be possible to determine qualitative as well as quantitative differences among the actinomycins. Actinomycins of differing chemical structures may be prepared by modifications of the culture medium. Modified actinomycins so produced retain antitumor activity but differ in toxicity. The possibility therefore exists that actinomycins may be found that possess improved therapeutic indices. It is also possible that some other form is active against certain neoplasms that at present do not respond to actinomycin therapy. The results presented in this paper indicate that the four ascitic tumor test systems may be used to differentiate qualitatively and quantitatively between the actinomycins tested.

Sugiura *et al.*[7] tested a large number of antibiotics against a spectrum of 20 mouse tumors, 5 rat tumors, and 1 hamster tumor. In their study no antibiotic was found to have antitumor activity against all tumors in the spectrum. A qualitative difference between actinomycins D and J was noted in the slight inhibition of Crabb hamster sarcoma by actinomycin J. Actinomycin D had no effect on this tumor. The authors state that ascitic tumors and the Ridgway osteogenic sarcoma of mice are the most useful tumors for studying actinomycins.

In an evaluation of the comparative chemotherapeutic activity of the actinomycins, the maximal effect obtainable must be taken into consideration. At present actinomycin II appears to give this effect in the Gardner lymphosarcoma ascitic tumor system. However, a 100 per cent cure rate, as determined by absence of grossly visible tumor and histological examination for tumor, was not obtained at any dose level tested.

It is possible that dosage regimens other than those used here will show increased effectiveness. In our studies, 3 successive daily doses were administered, starting 24 hours after tumor implantation. This regimen was highly effective with the Gardner lymphosarcoma system. However, Gregory *et al.*,[2] reporting from this laboratory on the ascitic sarcoma 180 system, showed that a total dose of 6 μg. actinomycin D (actinomycin IV), administered intraperitoneally over a period of 4 days, resulted in a greater extension of survival time than did the same total dose administered over a period of 2 or 3 days.

Under the conditions of the present study, the greatest effect against the sensitive tumors was obtained when a total of one fifteenth to one fortieth of the LD_{50} dose was administered. These results suggest that doses lower than the maximal tolerated dose might be included in the clinical testing of the actinomycins.

Summary

The cytostatic activities of actinomycins II, III, and IV on the following mouse ascitic tumor test systems were evaluated: Gardner lymphosarcoma, adenocarcinoma (S3A), Ehrlich Lettré hyperdiploid carcinoma, and sarcoma 180. The criterion of activity was an increase in survival time of treated mice over that of solvent-treated control mice. Of the four systems employed, the Gardner lymphosarcoma was found to be the most sensitive to the actinomycins. In this system, when the antibiotics were administered by the intraperitoneal route, the therapeutic index of actinomycin II was higher than that of actinomycins III and IV.

When tested at maximal tolerated doses, actinomycin II was not effective against the adenocarcinoma, Ehrlich carcinoma, or sarcoma 180; actinomycin III was not effective against Ehrlich carcinoma or sarcoma 180; actinomycin IV was effective against all four tumors.

Actinomycin IV was equally active against the Gardner lymphosarcoma when administered by intraperitoneal or intravenous routes of administration. The level of activity against the same tumor system after subcutaneous administration of actinomycins II and IV was lower than that observed after intraperitoneal administration.

The maximal effect of the actinomycins was attained with a total dose rang-

ing from one fifteenth to one fortieth of the LD_{50} dose when the actinomycins were administered intraperitoneally for 3 successive days. With this dosage regimen, occasional mice implanted wih the Gardner lymphosarcoma were free of tumor at the end of the test treatment period, as determined by gross and microscopic examination.

Acknowledgment

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BIOLOGICAL STUDIES OF ACTINOMYCIN D*

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In the summer of 1954, after scientific discussions between Selman A. Waksman and Sidney Farber, a preparation of actinomycin D, made available through the kindness of S. Waksman,[1] was subjected to preliminary trial in the Mouse Bioassay Unit of The Children's Cancer Research Foundation. An evaluation of results soon indicated that this substance possessed activity of a high degree against many of the rodent tumors routinely utilized at that time (TABLE 1).[2]

The tumors most favorably influenced were: P1534, a lymphatic leukemia in the DBA/2 mouse, DBRB, a mammary adenocarcinoma in the DBA/1 strain, another mammary adenocarcinoma, BCA in the C3H mouse, and the Cloudman malignant melanoma S91, also in the DBA/1 mouse. In all of these a marked to complete antitumor activity could be demonstrated.

Route of administration in all the early experiments was subcutaneous and the dose level usually 75 μg./kg., occasionally 60 and 100 μg./kg., daily. With the leukemias, therapy was begun 24 hours after tumor implantation; with the solid tumors, after a measurable tumor had been established.

Complete inhibition of tumor growth was obtained by this agent in some of the leukemias,[3] as shown in a section of P1534 implant (FIGURE 1). With other tumors there were also striking changes—from deceleration of tumor growth with more opportunity for differentiation, to necrosis of large masses of tumor cells, complete disappearance of tumors, or both. Occasionally, calcium deposition and cholesterol cleft formation were the only remnants of previous tumor, so absolute was the regression. This was particularly true of DBRB and Ridgway's osteogenic sarcoma, where extended survival without tumor was not uncommon. One hesitates to call these "cures," but it is hard to classify them otherwise.

Later trials with the agent utilized the intraperitoneal route, with the results shown in TABLES 2 and 3. In these experiments the actinomycin D used was

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obtained from Lederle Laboratories.* Manner of preparation and concentration at which the actinomycin D was used against the mouse tumors, indicated in the two tables, was identical. Ultraviolet spectra showed no qualitative or quantitative differences. In spite of these attempts to exactly duplicate the situation in both series of screening experiments, the 1958 preparation produced lesser tumor inhibition and greater toxic manifestations. This is a phenomenon that we have encountered repeatedly and that we cannot satisfactorily explain.

TABLE 1
ACTINOMYCIN D (JF1) VERSUS TRANSPLANTABLE RODENT TUMORS
(JULY 14, 1954 TO DECEMBER 9, 1954)

Tumor*	Dosage		Route	Evaluation	
	Level (µg./kg.)	Days		Survival	Tumor inhibition
Leukemia					
P1534 (6)	75	13–28	S.C.	±	+ to +++
C1498 (1)	75	13	S.C.	Sacrificed	++
AK4 (1)	75	8	S.C.	Sacrificed	
(1)	100	8	S.C.	Sacrificed	+ to ++
Mammary adenocarcinomas					
DBRB (1)	75	53	S.C.	+++	++ to +++
BCA (1)	75	20	S.C.	Sacrificed	+++
C3HBA (2)	75	8–15	S.C.	Sacrificed	+
E0771 (1)	75	9	S.C.	Sacrificed	±
Malignant melanoma					
S91 (5)	75	6–19	S.C.	–	± to ++
Sarcoma					
S180 (2)	75	7	S.C.	Sacrificed	– to ±
S37 (1)	75	8	S.C.	Sacrificed	–
713 (1)	75	8	S.C.	Sacrificed	±
T241 (1)	75	8	S.C.	Sacrificed	–
Miscellaneous					
6C3HED (1)	75	7	S.C.	Sacrificed	–
C954 (1)	75	7	S.C.	Sacrificed	±
C1300 (1)	75	10	S.C.	Sacrificed	– to ±

* Numbers in parentheses indicate numbers of experiments on which results were based.

A comparison, utilizing one sample given by two different routes (subcutaneous and intraperitoneal) is illustrated in TABLE 4. The results indicate that the subcutaneous route is the preferred one for the mouse, because of a greater increase in survival without loss of tumor-inhibiting properties. This may point to a possible mechanism, operating in the host that separates these two properties.

The LD_{50} varies with different actinomycins and with different samples of the same preparation (TABLE 5). Actinomycin B is by far the most toxic. A comparison of tumor-inhibition properties also shows variations, as illustrated in TABLE 6. Again, actinomycin B is distinguished by its marked toxicity

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* Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y. The material used in both series of screening experiments was from preparation 1687C-41A, and solutions were always prepared from this one source.

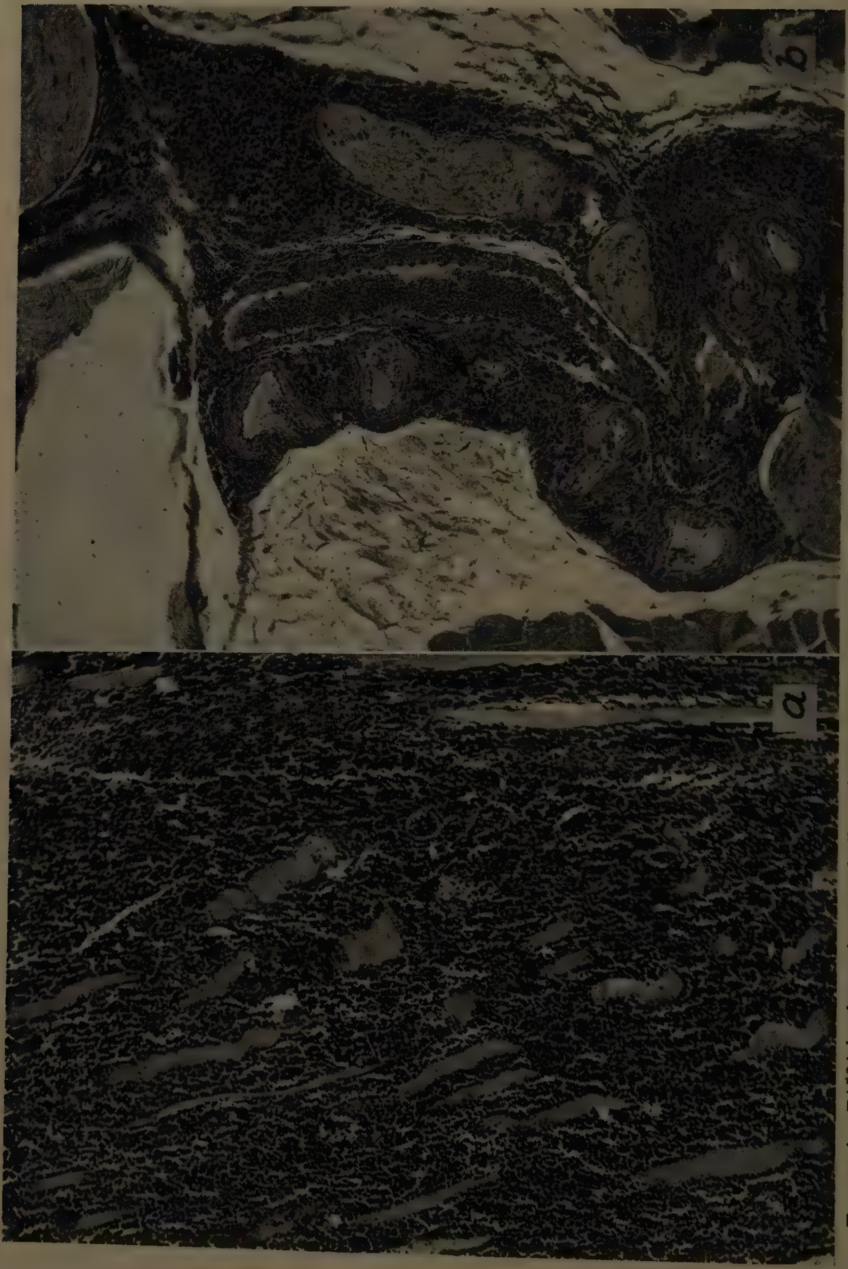

FIGURE 1. P1534 implant: *A*, control; *B*, actinomycin D-treated animal. Implant site intramuscular, at 18 days. ×125.

TABLE 2

ACTINOMYCIN D (JF3L) VERSUS TRANSPLANTABLE RODENT TUMORS
(FEBRUARY 27, 1957 TO OCTOBER 21, 1957)

Tumor	Dosage		Route	Evaluation	
	Level (μg./kg.)	Days		Survival	Tumor inhibition
Leukemia					
L1210	80	10	I.P.	±	
P1534	80	16	I.P.	±	+
C1498	20	21	I.P.	–	±
	80	15	I.P.	Toxic*	++
Mammary adenocarcinoma					
DBRB	80	12	I.P.	–	+++
Sarcoma					
S180	80	11	I.P.	–	±

* Survival time 25 per cent less than that of controls.

TABLE 3

ACTINOMYCIN D (JF3L) VERSUS TRANSPLANTABLE RODENT TUMORS
(JANUARY 9, 1958 TO FEBRUARY 18, 1958)

Tumor	Dosage		Route	Evaluation	
	Level (μg./kg.)	Days		Survival	Tumor inhibition
Leukemia					
L1210	80	13	I.P.	±	
P1534	80	12	I.P.	–	+++
C1498	80	11	I.P.	–	++
Mammary adenocarcinoma					
DBRB	5	14	I.P.	–	±
	20	15	I.P.	–	–
Sarcoma					
S180	5	11	I.P.		–
	20	13	I.P.	All toxic*	–
	80	10	I.P.		+

* Survival time 25 per cent less than that of controls.

TABLE 4

ACTINOMYCIN D (JF3L) VERSUS DBRB MAMMARY CARCINOMA
A Comparison of Subcutaneous and Intraperitoneal Administration

Dose and route		Survival time		Tumor inhibition*		
		Mean (days)	Increase (%)	Day 8	Day 11	Day 13
Control	No treatment	14.4				
40 μg./kg.	S.C.	28.6	+99	+100 (5)	+56 (5)	+26 (5)
40 μg./kg.	I.P.	15.0	+4	+71 (4)	+76 (4)	+35 (4)
80 μg./kg.	S.C.	24.4	+69	+100 (5)	+100 (5)	+100 (5)
80 μg./kg.	I.P.	10.2	–29	+100 (3)	Dead 4/5	Dead 4/5
160 μg./kg.	S.C.	7.6	–47	+100 (2)	Dead	Dead
160 μg./kg.	I.P.	5.2	–64	Dead	Dead	Dead

* Numbers in parentheses indicate number of mice on which tumor measurements were based.

coupled with slight to nonsignificant tumor inhibition. The 1958 Lederle actinomycin D sample is again more toxic than any of the other preparations (except, of course, actinomycin B).

The histopathological changes in mice become clear-cut when large doses—for example, 750 μg./kg. × 1 I.P.—are given. Such changes appear to be limited to the hematopoietic system, the gastrointestinal tract, and the gonads. Thus in the spleen (FIGURE 2) there is almost complete depletion of pulp cells with marked diminution in size of splenic follicles; in the bone marrow (FIGURE 3) an almost total aplasia is seen, and the small intestine (FIGURE 4) is characterized by atypia and plugging of glands by desquamated epithelium. Denudation of tips of villi may be seen in appropriate portions of such sections. With intraperitoneal administration, and occasionally at high doses with subcutaneous administration, changes in adipose tissues occur, namely, serous atrophy and fat necrosis (FIGURE 5). This appears to be due to contact of the agent (in concentrated solution) with fat, and may be due to a lipolytic-like activity, which we have not further investigated and which probably is not related to its destructive action on rapidly proliferating tissues.

TABLE 5

LD_{50} (FIVE-DOSE SCHEDULE) OF ACTINOMYCINS

Agent and source	Route	Dose (μg./kg.)
Actinomycin B (Pfizer)	I.P.	50
Actinomycin C (Pfizer)	I.P.	>400
Actinomycin C (Bayer)	I.P.	<500
Actinomycin D (Lederle)	I.P.	<300

TABLE 6

TUMOR INHIBITION OF ACTINOMYCINS

Agent and source	P1534 leukemia			DBRB mammary cancer		
	Dose*	Survival	Tumor inhibition	Dose	Survival	Tumor inhibition
	(μg./kg./day)	Increase (%)	(%)	(μg./kg./day)	Increase (%)	(%)
Actinomycin B (Pfizer)	5	+13	+35	1.25	−13	+13
Actinomycin C (Pfizer)	80	+6	+100	80	−11	+100
Actinomycin C (Bayer)	80	+22	+76	80	−22	+36
Actinomycin D (Lederle, 1957)	80	+20	+65	80	−14	+100
Actinomycin D (Lederle, 1958)	80	−23	+100	5	Toxic	+31

* Intraperitoneal route.

An enhancement effect, occurring when actinomycin D in relatively ineffective doses is given with X irradiation, has been studied in both normal and malignant tissues of the mouse. Such treatment was given to confirm results of a similar nature obtained in man.[4]

When normal mouse skin is subjected to both radiotherapy and actinomycin D (the latter administered parenterally and hence incapable of producing a local skin reaction), there is observed: (1) more mice reacting positively with erythema and loss of hair to the combined treatment than to X irradiation alone, (2) an earlier appearance of these reactions in the "combination" group, and (3) more intense reactions in the combination group.

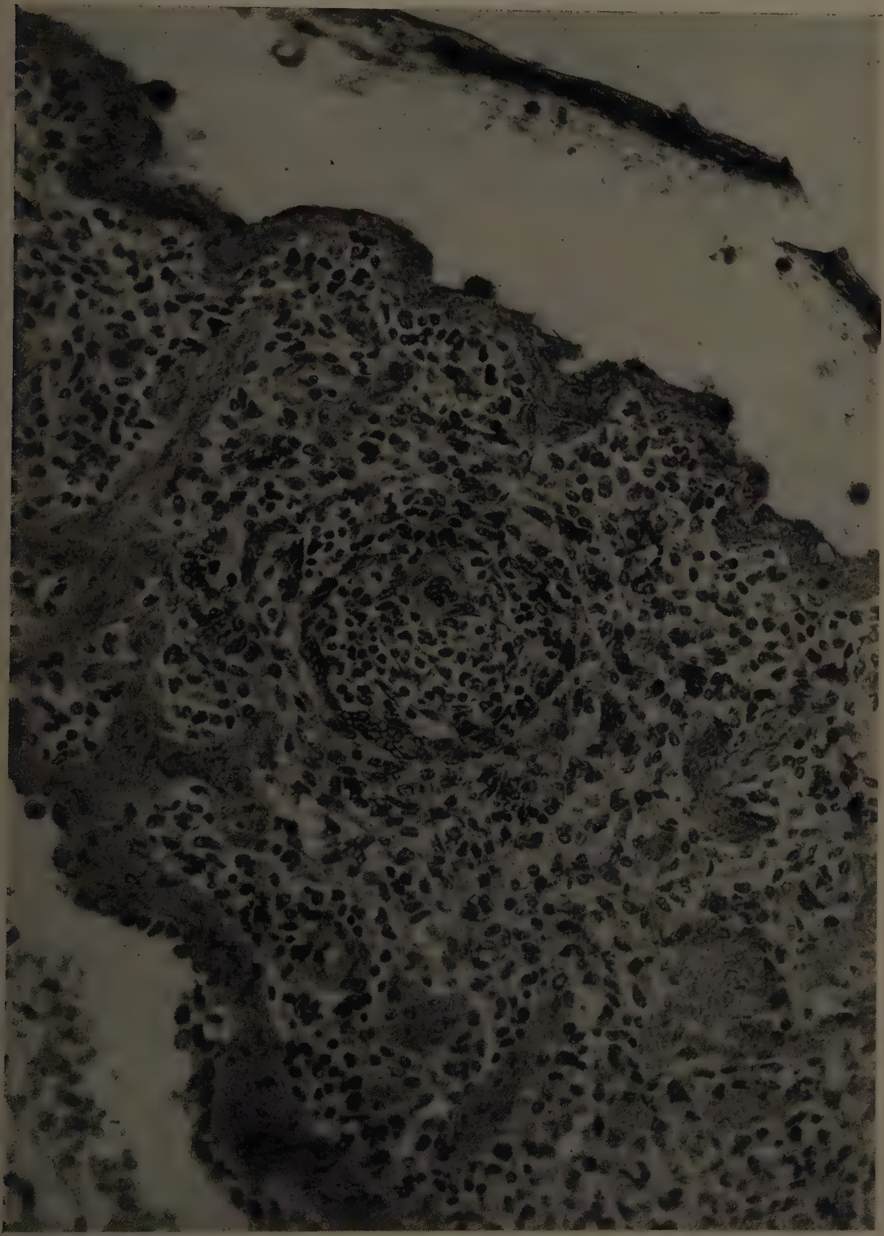

FIGURE 2. Mouse spleen after one intraperitoneal injection of actinomycin D at 750 μg./kg. Note marked depletion of pulp cells and diminution in size of follicle. ×425.

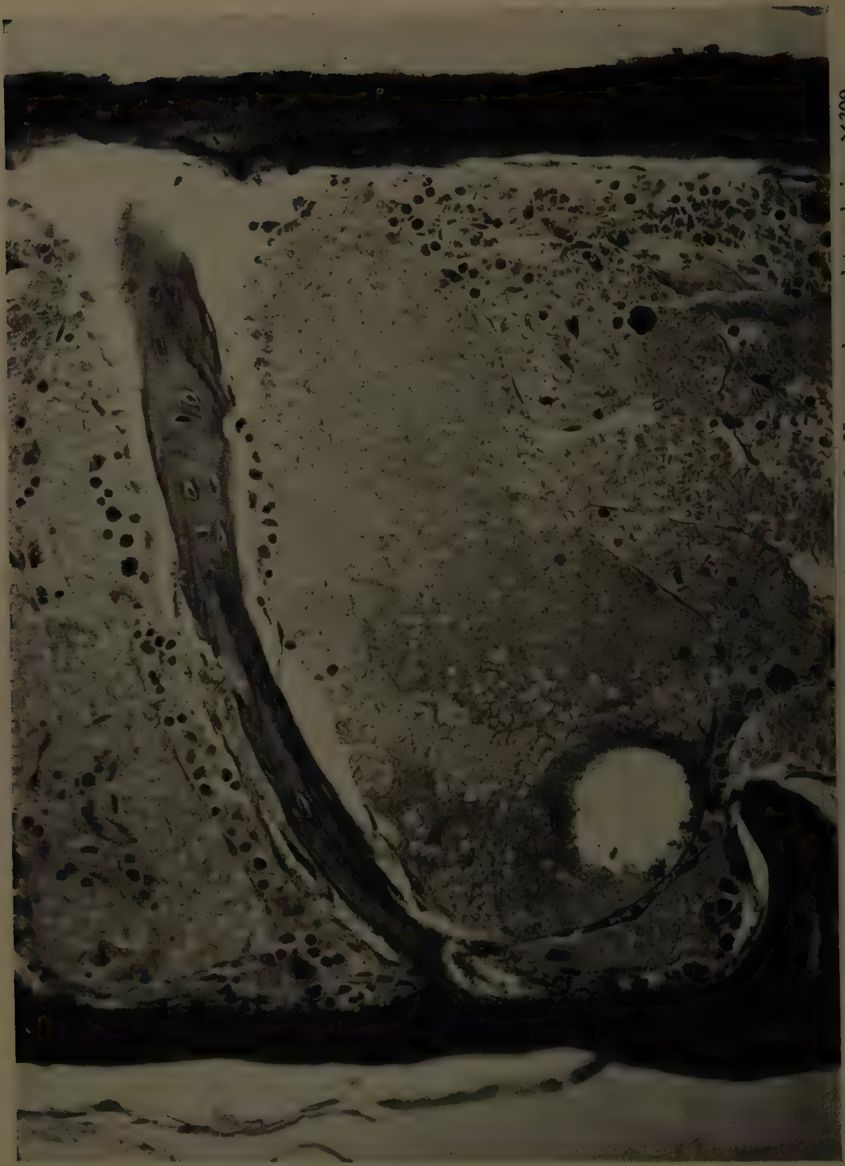

FIGURE 3. Mouse bone marrow, from same animal as in FIGURE 2. Note nearly complete aplasia. ×300.

To illustrate this, in one experiment (TABLE 7) actinomycin D was administered intravenously at 150 μg./kg. and X rays were given at the two dose levels of 300 r and 1000 r to the right hind leg of CAF/Jax mice, the animals being shielded from the direct beam and back-scatter by lead shielding (radiation

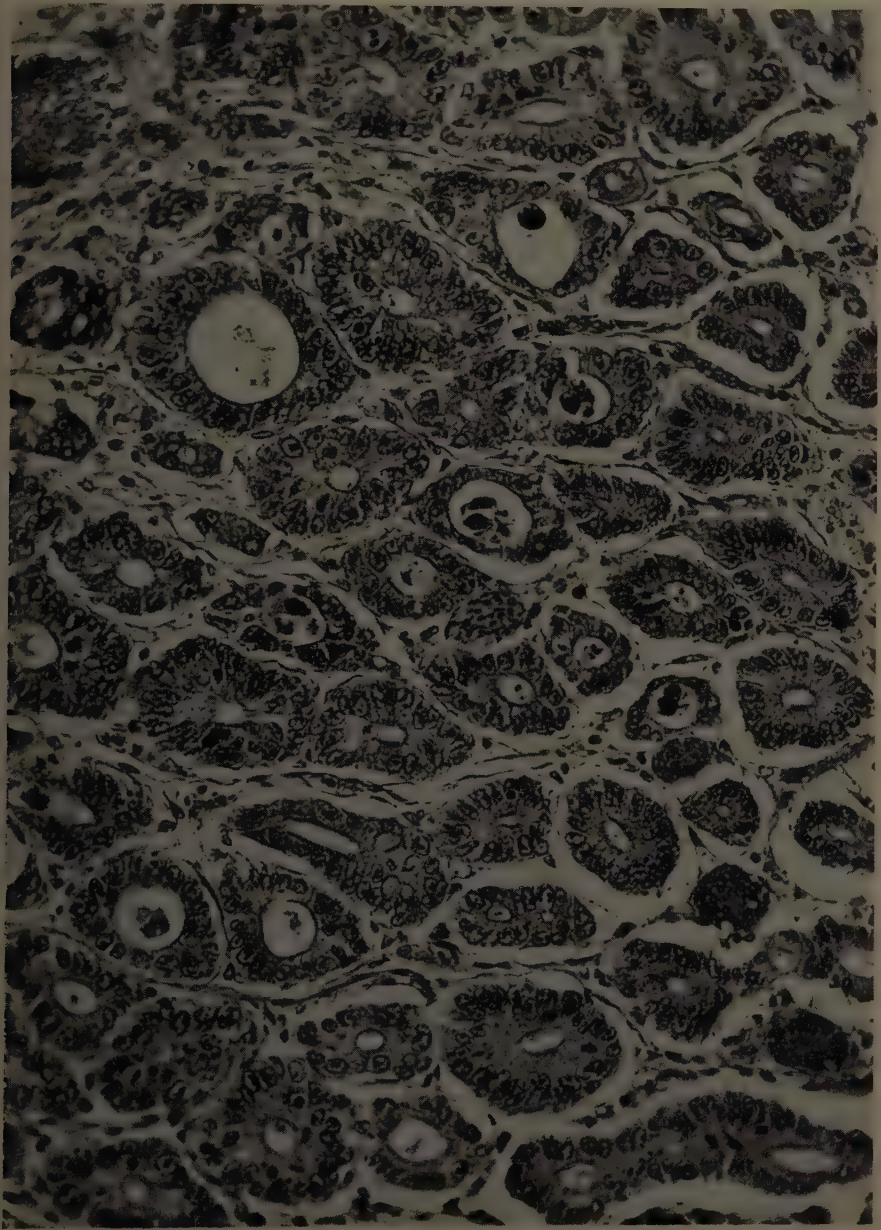

FIGURE 4. Mouse duodenum, from same animal as in FIGURE 2. Note cell detritus in glands. ×550.

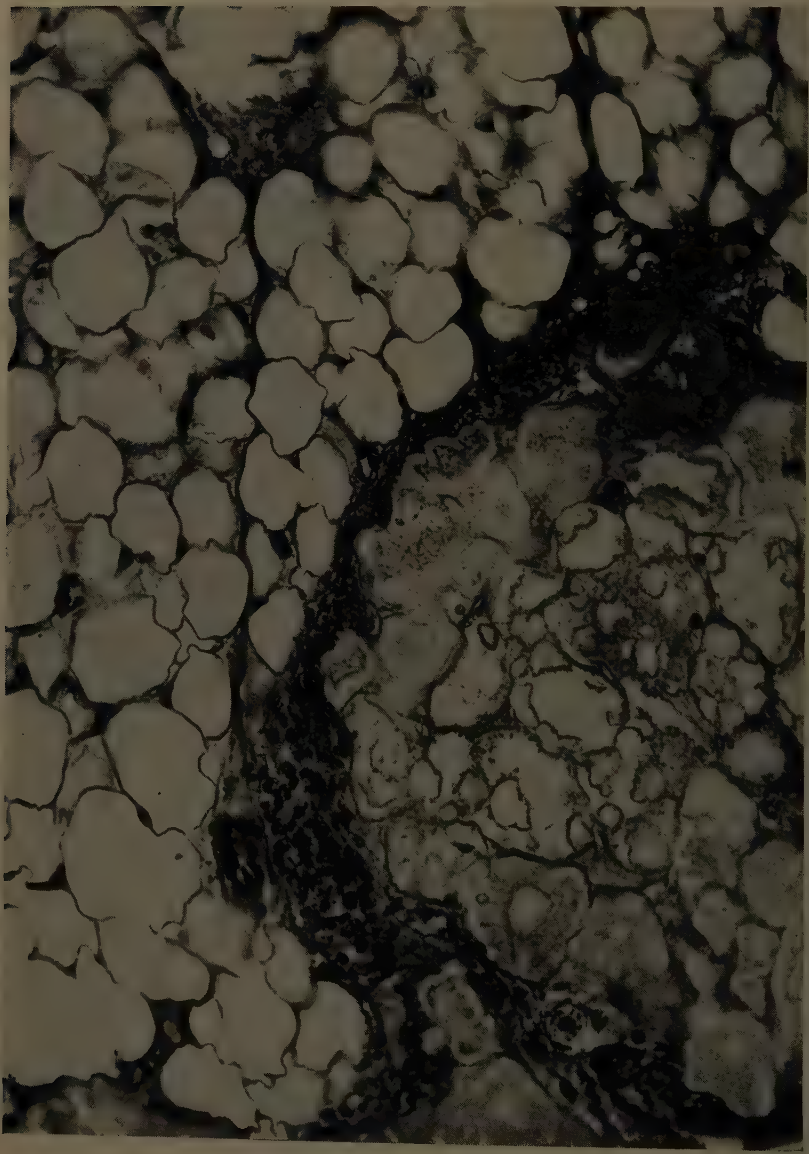

FIGURE 5. Mesenteric fat of mouse after five intraperitoneal injections of actinomycin D at 300 μg./kg. Note serous atrophy and necrosis. ×550.

factors being 250 kv [constant potential], 15 ma, 40 cm. T.S.D., 1 mm. Al [added filtration] HVL 0.63 mm. Cu). The differences in the various columns are obvious. The total number of positive reactions in combination groups, compared with that of the X-ray groups, is very much increased. This is true also of the average number of positive responses per reacting mouse. When methotrexate or mitomycin C is substituted for actinomycin D in similarly designed experiments, there is no evidence of enhanced reaction. Enhancement of X-ray effects can be demonstrated for other normal tissues. Thus with appropriate doses and exposures one can show a greater degree of injury to both hematopoietic tissue and the small intestine when actinomycin D, at doses that have no observable effect on these tissues (histologically evaluated), is added to a relatively innocuous dose of X ray. Such a combination, when the target is the small intestine may result in death of the animal.

The same phenomenon of enhancement may be demonstrated in transplantable tumor systems. To best illustrate it, one must have a tumor that is responsive to either or both of the agents used. The Ridgway osteogenic sarcoma in the AKR mouse is such a system (FIGURE 6). This enhancement of the effects of X ray, relative to its destructive action on malignant tissue, is not a unique property for actinomycin D, but it is one that has been noted by a number of investigators, for example, Heidelberger,[5] for 5-fluorouracil, and G. J. D'Angio, C. L. Maddock, and B. Brown (unpublished observations, 1959), for mitomycin C. However, with the dose level of mitomycin C employed, which was 2 mg./kg. for three successive days, the outcome, instead of being extended to indefinite survival, was a somewhat prolonged one, terminating in death due to a delayed toxicity of the antibiotic.

TABLE 7

POTENTIATING EFFECT OF ACTINOMYCIN D ON RESPONSE OF NORMAL MOUSE SKIN TO X RAY*

Dosage	No. of + observations/ total no. of +-reacting mice	Day of earliest + change	Mice reacting on day 12 (%)	Average no. responses per +-reacting mice
300 r (5)†	3/2	15	0	1.5
1000 r (5)	18/2	13	0	9.0
Actinomycin D (5)‡	0/0	—	0	0.0
300 r + actinomycin D (15)	68/3	10–12	26.7	5.2
1000 r + actinomycin D (15)	235/15	6	93.3	15.7

* Total period of observation was 42 days.

† Nos. in parentheses denote numbers of mice in category.

‡ All groups receiving actinomycin D given agent at 150 μg./kg. I.V. × 1.

We have made some abortive attempts at elucidating the mechanism of action of actinomycin D, as regards its tumor-destroying property but, in contradistinction to a competitive reversal of effect by calcium pantothenate demonstrated by Foley[6] in the microbiological systems *Lactobacillus arabinosus* and *L. fermenti*, this has not been true in the DBRB tumor system, using a 64-fold range of concentration of calcium pantothenate (TABLE 8). The DBRB tumor system, since it always responds to actinomycin D therapy

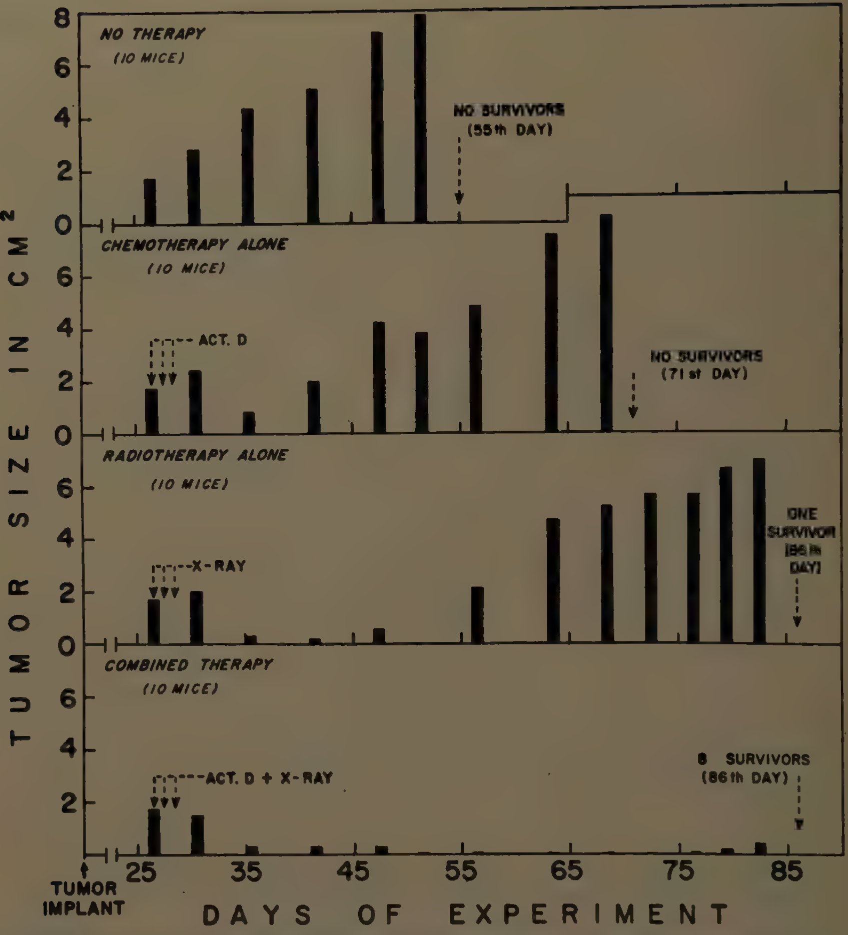

FIGURE 6. Effects of combined chemo- and radiotherapy; tumor size *versus* days of experiment. Agents: actinomycin D (75 μg./kg. I.P. ×3) and X ray ($400_{[\mathrm{air}]}$ ×3). Tumor: Ridgway osteogenic sarcoma in AKR mice.

TABLE 8

FAILURE OF CALCIUM PANTOTHENATE TO REVERSE ANTITUMOR EFFECT OF ACTINOMYCIN D ON DBRB MAMMARY CARCINOMA

Treatment		Survival		Tumor inhibition (%)	
Actinomycin D (μg./kg.)	Ca pantothenate (mg./kg.)	Mean (days)	Increase (%)	Day 11	Day 13
0	0	12.0			
80	0	23.4	+95	+81	+49
80	6.25	20.2	+68	−9*	+42
80	25	21.6	+80	+66	+44
80	100	20.0	+67	+83	+44
80	400	23.4	+95	+56	+49

* An initial sudden increase in tumor growth, especially in one mouse, was responsible for this irregularity. The mouse died before the 13th day tumor measurements were obtained.

(subject to some variations mentioned previously) would seem to be a good choice for the demonstration of this effect. However, even with actinomycin D given at its most effective dose, and by a subcutaneous route, which is tolerated over the longest period of time, no reversal could be demonstrated.

Our work with actinomycin D at The Children's Cancer Research Foundation has not been limited to one kind of experimental animal. The drug's effect on several rat leukemias (WR-2, WR-3, and WR-6) in the Furth inbred strain of Wistar rats has not been outstanding. Toxicity studies also have indicated that the rat is more sensitive to undesirable side reactions. When administered intraperitoneally, actinomycin D produces considerable irritation within the peritoneal cavity, with subsequent adhesions as therapy is continued.

Actinomycin D, at maximal tolerated doses, has produced some striking effects when used against heterologous tumors grown in the hamster cheek pouch.[7] Utilizing the agent at the maximal tolerated dose of 125 μg./kg. intraperitoneally administered for seven to eight days, tumor tissue is reduced to a mere nubbin in both ME-1 and MFS-1. These are both tumors of human origin, the one an amelanotic melanoma from an adult, the other a myxofibrosarcoma from a child.

Heterologous tumors of nonhuman origin also respond to actinomycin D, for example, the P1534 from the mouse, and two derived from tissue culture lines, KB and S180. Enhancement of the effect produced by combining X irradiation with actinomycin D can also be demonstrated in heterologous tumors grown in the hamster cheek pouch. This effect is, therefore, far from unique for one laboratory animal, the mouse.

Summary

We have made the following observations as a result of our experience with actinomycin D.

Actinomycin D has shown marked antitumor activity against many transplantable mouse tumors used routinely for screening purposes, particularly against P1534, DBRB, and S91.

Occasional complete regressions with extended survival have been observed with DBRB and Ridgway's osteogenic sarcoma.

Antitumor activity has also been marked in heterologous tumors, notably ME-1 and MFS-1 grown in the hamster cheek pouch.

Better results, in terms of greater antitumor activity and diminished toxicity, have been noted in the mouse when the agent is administered by the subcutaneous rather than the intraperitoneal route.

Manifestations of toxicity appear to be limited to (a) the hematopoietic system (bone marrow and spleen), (b) the gastrointestinal tract, particularly the small intestine, and (c) the gonads, concerning which our experience is exclusively with male animals.

At both low and high doses, local manifestations of toxicity are represented by the usual inflammatory reaction. At high doses administered intraperitoneally, an unusual reaction, which includes serous atrophy and fat necrosis, may be seen in adipose tissue.

The agent can enhance the response to irradiation of both normal and malignant mouse tissue. This is more than a simple additive reaction. The same phenomenon has been noted in heterologous malignant tissue grown in the hamster cheek pouch.

There is evidence that actinomycin B differs significantly from actinomycins C and D, both in toxicity and antitumor activity, and that these properties do not necessarily vary in the same direction.

Calcium pantothenate, in one tumor system, has been incapable of reversing the antitumor effect of actinomycin D.

There seems to be some lack of reproducibility of both toxic and antitumor properties of different samples of actinomycin D, which is not satisfactorily explained by the usual variables in biological experiments.

Acknowledgments

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THE EFFECT OF ACTINOMYCINS AND THEIR DERIVATIVES ON A SPECTRUM OF TRANSPLANTED MOUSE LEUKEMIA*

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Actinomycins, first isolated from a culture of *Streptomyces antibioticus* by Waksman and Woodruff,[1] have been shown by Stock,[2] Reilly *et al.*,[3] Hackmann,[4-6] Farber *et al.*,[7,8] and other investigators to inhibit the growth of experimental animal tumors. Subsequently activity has been demonstrated also against neoplastic diseases in man. Schulte[9-11] reported actinomycin C to be effective against Hodgkin's disease, and Farber *et al.*[7,8,12] noted the activity of actinomycin D against Wilms's tumor, rhabdomyosarcoma, and Hodgkin's disease. Others have confirmed these results and continued their studies of patients with cancer.[13-33] The actinomycins now are of definite interest in cancer chemotherapy.

Analysis by Brockmann and his associates[34] and by Bullock and Johnson[35] has shown that the actinomycin molecule consists of two polypeptide chains linked to a chromophore group, 2-aminophenoxazine-3-one. As an example, the structural formula of actinomycin C_3, as presented by Brockmann *et al.*,[34] is shown in FIGURE 1. Differences in this basic structure have been found or induced, a brief listing of which follows:

Changes in the peptide moiety:

(1) Different actinomycins have been obtained from cultures of a number of *Streptomyces* species.[36-39] The designations A, B,[40-44] C,[45,46] D,[47] I,[48,49] J,[50,51] and X[48,49] have been used to classify them according to certain differences in their properties, which reflect variations in number, arrangement, and types of amino acids in the peptide chains. Each of these substances contains a mixture of closely related compounds, as demonstrated for actinomycin C first by Brockmann and Grubhofer[52] and Brockmann and Pfennig.[53]

(2) The particular nitrogen source (nitrate, nitrite, or amino acids) supplied to the culture medium has been shown to influence the composition of the actinomycin complex synthesized by a given organism.[48,53-55] Schmidt-Kastner[56] has reported the production of new actinomycin complexes (E and F) by growing cultures when DL-isoleucine or sarcosine was added.

(3) The peptide chains have been removed by hydrolysis and the resulting despeptidoactinomycin (chromophore) has been shown to be identical for actinomycin C_2, C_3, X_2, I_1, and B.[57,58]

Changes in the chromophore group: Brockmann has chemically altered this part of the molecule by removing the amino group[59] or by substituting one or both hydrogens of the amino group with different side chains.†

The question then arises whether these compounds would differ in their

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† H. Brockmann, personal communication, 1959.

antineoplastic activity. Clarke observed a significantly better activity of actinomycin F_1 (KS4) than of actinomycin D against a spontaneous mammary carcinoma of the mouse.* Hackmann and Schmidt-Kastner found activity of actinomycins E_1, E_2, F_{0-5}, C, and D against experimental tumors with large differences in maximal tolerated doses.[6] Pugh *et al.* tested actinomycins C and D and components of the A, B, and D complex for activity against Gardner lymphosarcoma (6C3HED) and adenocarcinoma (C3HBA) in mice.[60] There was significantly lower activity of the components A_v and B_v.

We have studied the effects of a number of actinomycins on transplanted mouse leukemias in the hope of selecting, for clinical trial, compounds more promising than those presently used in patients. The results of the studies are herewith reported.

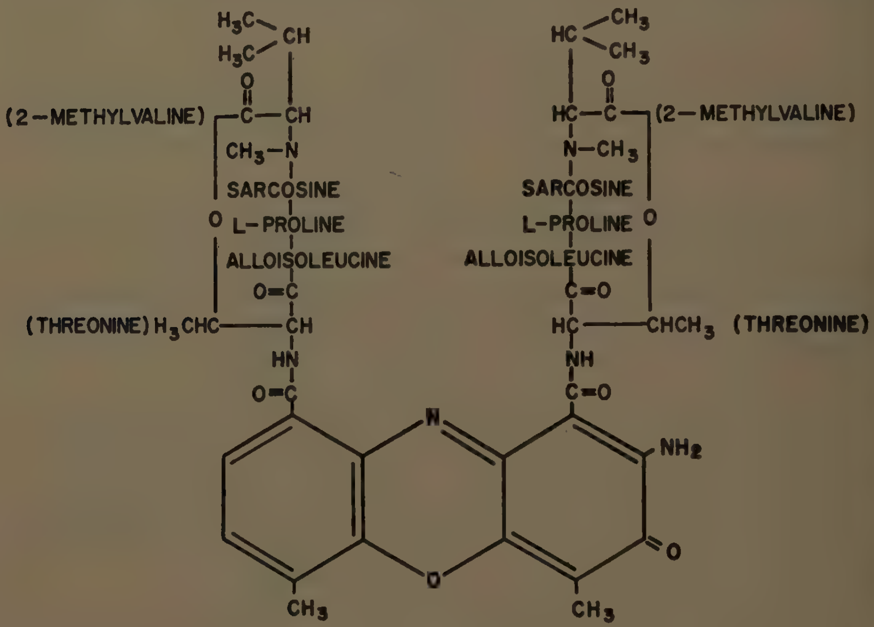

FIGURE 1. Actinomycin C_3.

Materials and Methods

The technique used in this laboratory for evaluation of the chemotherapeutic activity of a given drug by its ability to prolong the survival time of mice with transplanted leukemia has been reported previously.[61] In this study, leukemias L1210,[62] P1081,† P815,[63] P388,† and P329,† carried in DBA mice or F_1 hybrids thereof, have been used. Experiments measuring the inhibitory effect on the local growth of subcutaneously injected leukemic cells have been done with leukemia B82,[64] carried in F_1 hybrids of the Bagg albino × C58 cross. In this case, the mice were killed 12 to 14 days after inoculation and the subcutaneous tumors dissected out and weighed. In some experiments, leukemia B82 was inoculated intraperitoneally, causing generalized leukemia, and survival time was measured. The compounds* were dissolved in 0.9 per cent saline or in ethyl alcohol plus 0.9 per cent saline, with or without Tween 80, and kept protected from light. The dilution was such that the volume to be given at one injection was 0.01 ml./gm. mouse. Usually, the animals were injected intraperitoneally six or three times a week for a total of 10 or 5 injections respectively. In a small number of earlier experiments with generalized leukemia, treatment was continued until the animals died.

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* D. A. Clarke, personal communication, 1958.
† M. Potter, personal communication.

TABLE 1
AMINO ACID COMPOSITION OF VARIOUS ACTINOMYCINS

Amino acid composition (moles/mole) of actinomycin, mol. wt. 1200	B			C			D	E		F					I		X		
	B_1	B_4	B_5	C_1	C_2	C_3	D_4	E_1	E_2	F_1	F_2	F_3	F_4	F_5	I_0	I_1	$X_{0\beta}$	X_1	X_2
L-Threonine	2	2	2	2	2	2	2	+	+	+	+	+	+	+	+	+	+	2	2
Sarcosine	2	2	2	2	2	2	2	+	+	+	+	+	+	+	+	+	+	2	2
L-Proline	1	2	1	2	2	2	2	+	+	−	+	−	+	+	+	+	+	2	1
D-Valine	2	2	2	2	1	0	2	−	−	+	+	−	−	+	+	+	+	2	2
N-CH_3-L-Valine	2	2	2	2	2	2	2	+	−	+	+	+	+	+	+	+	+	2	2
D-Alloisoleucine				0	1	2		+	+	+	+	+	+	+	0	0	0	0	0
Hydroxyproline				0	0	0		−	−	−	−	−	−	−	0	0	+	0	0
N-Methylisoleucine								+	+	−	−	−	−	−					
Relative percentage of components in complex	9.6	28.1	59.3	18.1	45.7	34.4	100								7.3	86.5	5.1	5.1	88.6

Results

In order to facilitate understanding, a brief description of the amino acid distribution for different actinomycins used in our studies and the composition of the actinomycin complexes are given in TABLE 1, which quotes data of Brockmann and Gröne,[49,65] Brockmann and Pampus,[66] Roussos and Vining,[67] and Schmidt-Kastner.[56] Minimal fractions of some complexes are omitted from this table. TABLE 2 summarizes the results of our experiments with actinomycins produced by different species of *Streptomyces* (B+C, C_1, C_2, C_3, D, and I) or by a change in amino acid composition of the medium (E_1, E_2, F_1, F_2, F_3, F_4, and F_5) against leukemias L1210, P1081, B82, and P388. Actinomycins D and F_1 (KS4) have been tested most thoroughly. Because of inadequate supply, only a limited number of tests could be performed with the remaining compounds. There appear to be no striking differences in activity of the different agents against the leukemia spectrum.

* Supplied by Hans Brockmann, Organisch-chemisches Institut der Universitaet Goettingen, Germany: Farbenfabriken Bayer AG, Wupperthal-Elberfeld, Germany; Hoffmann-LaRoche Inc., Nutley, N. J.; Merck Sharp and Dohme, Research Laboratories, Division of Merck and Co. Inc., Rahway, N. J.

Only a minor degree of activity has been observed on leukemia L1210 and a slightly better effect on leukemia P388. Leukemias B82 and P1081 showed good response. There were considerable differences, however, in the optimal dose levels. In this respect, these compounds can be divided roughly into three groups. Of the actinomycins B + C, C_1, C_2, C_3, D, E_1, F_5, and I, the mice tolerated 0.2 mg./kg. injected intraperitoneally three times weekly. Of F_1 (KS4) and F_3 a tenfold dose could be given. The maximal tolerated doses of E_2, F_2, and F_4 were found in between. TABLE 1 shows that F_1 and F_3, tolerated at the highest dose, are the only actinomycins thus far known to contain no L-proline. F_4, tolerated at 0.8 mg./kg. three times weekly, is lacking in D-valine. E_1 and E_2, also devoid of this amino acid, were toxic at lower doses; both, however, contain *N*-methylisoleucine, which is not present in F_4. The actinomycins of the X complex are not listed in the table, because the amounts available were not sufficient for adequate tests. The maximal tolerated doses were more than 0.4 mg./kg. three times weekly for X_0, 0.2 mg./kg. three times weekly for X_1, and 0.1 mg./kg./day for X_2. Of actinomycin $X_{0\beta}$,[66] we tested the monopalmitate, which was tolerated up to 2 mg./kg./day and produced 52 per cent inhibition of leukemia B82 at that dose. This compound is the only actinomycin containing hydroxyproline.

TABLE 2

EFFECTS OF ACTINOMYCINS ON TRANSPLANTED MOUSE LEUKEMIA

Actinomycin	Optim. dose (mg./kg. tiw × 5)	Leukemia			
		L1210*	P1081*	B82†	P388*
B + C	0.2			+	
C_1	0.15			+	±
C_2	0.2	±		±	±
C_3	0.2	±		+	±
D	0.2	±	+	+	+
E_1	0.2	±			+
E_2	0.4				±
F_1 (KS4)	2.0	±	+	+	±
F_2	0.4	±		+	±
F_3	2.5	±	+	+	
F_4	0.8	±			
F_5	0.2	±			+
J	0.2	±	+	+	+

* Increase of survival time: +, >59 per cent; ±, 25 to 59 per cent.
† Tumor inhibition: +, >79 per cent; ±, 40 to 79 per cent.

Of the actinomycins altered chemically by Brockmann, the chromophore group alone was toxic at 125 mg./kg. three times weekly. There was no antileukemic effect with the maximal tolerated dose of 62.5 mg./kg. Replacement by a hydroxyl group of the chromophore amino group or substitution of one or two hydrogens of this amino group resulted in agents with activity against the leukemia spectrum comparable to that of the parent compounds. In these experiments, leukemias L1210, P1081, B82, P388, and P329 have been used (TABLE 3). There were differences in response of the different leukemias, but no significant differences in the activities of the compounds against the same strain of leukemia. For derivatives of actinomycin C_3 this is shown in more detail in TABLE 4. Exceptions are paraphenylenediamino- and ethylenediaminoactinomycin C_3, which appeared to be less active (TABLE 3). Again, large differences in the maximal tolerated doses

TABLE 3
EFFECTS OF ACTINOMYCINS AND DERIVATIVES ON TRANSPLANTED MOUSE LEUKEMIA

Actinomycin	Optim. dose (mg./kg. tiw × 5)	Leukemia				
		L1210*	P1081*	B82†	P388*	P329*
D	0.2	±	+	+	+	+
F_1 (KS4)	2.0	±	+	+	±	+
C_2	0.2	±		±	±	
C_2, *N*-Ethyl-	1.0	±	+	+	±	
C_2, desamino-	30.0			+		+
C_3	0.2	±		+	±	
C_3, *N*-β-Hydroxyethyl-	3.0	±	+	+	+	+
C_3, *N*-methyl-	2.0	±	+	+	+	+
C_3, *N*-dimethyl-	4.0	±		+		+
C_3, *N*-dimethylene-	5.0	±	+	+	±	
C_3, *N*-β-diethylaminoethyl-	2.0	±	+	+		+
C_3, *N*-amino-	2.0	±	+	+		+
C_3, paraphenylenediamino-	120.0			±		±
C_3, ethylenediamino-	120.0			+		−

* Increase of survival time: +, >59 per cent; ±, 25 to 59 per cent.
† Tumor inhibition: +, >79 per cent; ±, 40 to 79 per cent.

TABLE 4
EFFECTS OF MAXIMAL TOLERATED DOSES OF ACTINOMYCINS ON TRANSPLANTED MOUSE LEUKEMIA (PER CENT INCREASE OF SURVIVAL TIME)

Actinomycin	L1210		P1081		P329	
	Δ Wt. (gm.)	Incr. surv. time (%)	Δ Wt. (gm.)	Incr. surv. time (%)	Δ Wt. (gm.)	Incr. surv. time (%)
	+2.6	—	+1.4	—	−1.5	—
C_3, *N*-amino-	−1.4	36	−0.4	178	−1.7	69
C_3, *N*-methyl-	−1.8	52	−0.1	193	−1.7	86
C_3, *N*-dimethyl-	−1.2	40	+0.2	128	−1.5	74
C_3, *N*-dimethylene-	−2.0	42	−0.4	149	−2.7	58
C_3, *N*-β-hydroxyethyl-	−1.6	38	−0.4	193	−2.1	82
C_3, *N*-β-diethylaminoethyl-	−1.7	40	−1.6	193	−1.2	70

were noted. None of the derivatives was active at doses equal to those of the parent compounds. The optimal doses were: *N*-ethylactinomycin C_2, 5 times that of the parent compound; *N*-methyl-, *N*-β-diethylaminoethyl- and *N*-aminoactinomycin C_3, 10 times; *N*-β-hydroxyethylactinomycin C_3, 15 times; *N*-dimethylactinomycin C_3, 20 times; and *N*-dimethyleneactinomycin C_3, 25 times. Of desaminoactinomycin C_2, only a 150-fold dose was fully effective. That 600-fold doses of paraphenylenediamino- and ethylenediaminoactinomycin C_3 were tolerated and were necessary to produce minor antileukemic effects suggests to us that these compounds are inactive and may have been contaminated with minimal amounts of more active substances.

So far, only differences of maximal tolerated doses, but no therapeutic superiority of one or the other compound, have been demonstrated. TABLE 5 shows an attempt to determine the dose response to actinomycins D and F_1 (KS4), *N*-β-hydroxyethylactinomycin C_3, and *N*-methylactinomycin C_3 of leukemia B82 grown as a subcutaneous tumor, thus to ascertain whether there would be differences in chemotherapeutic index of these compounds. From the data presented in TABLE 5 it appears that the ratio of the maximal tolerated (fully effective) dose to the dose producing 40 per cent inhibition was smaller than 2 for actinomycin D, between 2 and 3 for actinomycin F_1 (KS4), 3 for *N*-β-hydroxyethylactinomycin C_3, and close to 4 for *N*-methylactinomycin C_3. These figures are based on the assumption that the largest dose tolerated in this experiment was less that 0.2 mg./kg. for actinomycin D and less than 3 mg./kg. for *N*-methylactinomycin C_3, since such doses produced severe weight loss of all animals and early death of some.

TABLE 5

EFFECTS OF ACTINOMYCINS ON LEUKEMIA B82: DOSE RESPONSE

Compound	Dose (mg./kg. tiw × 5)	Δ Wt. (gm.)	Tumor inhib. (%)	Mort.	Chemotherap. index
Control	—	+3.4	—	0/10	
Actinomycin D	0.2	−4.2	90	5/10	<2
	0.1	−1.0	52	0/10	
	0.05	+1.7	25		
Actinomycin F_1 (KS4)	3	−3.7	88	0/10	
	2	−0.6	68	0/10	2
	1	+1.8	33		
	0.5	+2.5	31		
N-(β-Hydroxyethyl) Actinomycin C_3	3	−3.1	89	0/10	3
	2	−1.7	74	0/10	
	1	+1.7	49		
	0.5	+4.1	7		
N-Methyl Actinomycin C_3	3	−5.2	99	5/10	4
	2	−2.9	83	0/10	
	1	−0.3	65		
	0.5	+3.0	41		

Discussion

Actinomycin complexes produced naturally and their purified components, as well as derivatives made biosynthetically or synthetically, inhibit certain strains of transplanted mouse leukemia. There are no clear-cut qualitative differences in activity against any of the leukemia spectrum for the different compounds tested, with the exception of ethylenediamino- and paraphenylenediaminoactinomycin C_3 and the chromophore group, which have little or no antileukemic activity, after removal of the peptide chain. Quantitative differences of activity exist, however, and are dependent on the amino acid compositions of the peptide chains or on changes concerning the chromophore amino group. Maximal tolerated doses differ greatly, but the inhibitory effect produced by these doses is equal. Against the leukemia B82, actinomycin F_1 and *N*-β-hydroxyethyl- and *N*-methylactinomycin C_3, perhaps have some advantage in chemotherapeutic index over actinomycin D. Such differences, although slight, suggest the importance of studying for their clinical value some of the actinomycins substituted at the chromophore amino group.

With the increasing number of actinomycin derivatives studied experimentally, the need for a system of descriptive nomenclature becomes urgent, and no satisfactory one yet has been provided. We do not believe that the problem can be solved by assigning letters or numerals to the compounds on a more or less arbitrary basis. Abbreviated names derived from the chemical terminology seem preferable and would facilitate greatly the correlation of structure and biological activity.

Summary

A variety of actinomycins and actinomycin derivatives has been tested against a spectrum of transplanted mouse leukemias.

Changes in the peptide moiety produced as much as a tenfold variation of maximal tolerated doses.

The chromophore group alone was inactive at toxic levels.

The desamino derivative of actinomycin C_3 was active at a dose 150 times the dose of the parent compound.

Derivatives of actinomycin C_2 and C_3 substituted at the chromophore amino group were active at a dose 5 to 25 times the dose of the parent compound.

Some of these derivatives may have a slightly better chemotherapeutic index against leukemia B82 than actinomycin D. Clinical trial is suggested.

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EXPERIMENTAL EVALUATION OF ACTINOMYCIN D*

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Antineoplastic activity of actinomycin D ranges from dramatic to slight. Mouse ascites tumors were greatly inhibited in studies of Sugiura (1956) and Gregory *et al.* (1956). A slight antineoplastic activity against sarcoma 180 (S180) has been reported by Sugiura (1956) and Reilly *et al.* (1953), and against malignant melanoma S91, mammary adenocarcinoma, and both myeloid and lymphoid leukemia, by Farber (1955). According to Fantani *et al.* (1956), a glioblastoma, S91 melanoma, and C3H mammary adenocarcinoma showed the greatest sensitivity to actinomycin D. Following partial hepatectomy, inhibition of hepatic regeneration in the rat and, to some extent, in the mouse was demonstrated by Garattini *et al.* (1956), who also studied actinomycin D in combination with local irradiation, desacetyl methylcolchicine, Carzinophilin, and sarcolysine in rodent tumor systems. DiPaolo *et al.* (1957) found the antibiotic most effective against mammary adenocarcinomas C755 and C3HBA, and Krebs 2 ascites carcinoma. Complete disappearance of some solid lymphomas resulted from single massive intraperitoneal injections. Several courses of treatment with actinomycin D successfully controlled an ascites associated with a spontaneous ovarian carcinoma in a dog.

Maximal tolerated dosages of actinomycin, like the alkylating agents and, in some respects, like X rays, cause loss of spleen weight and atrophy of the thymus and lymph nodes. Direct contact with the skin results in deep ulcers (DiPaolo *et al.*, 1957).

The experiments reported here represent part of an effort to evaluate the properties of actinomycin D. Included are studies of the antineoplastic activity of actinomycin D and of five phenoxazinones, the dose-response curve of a sensitive tumor, combination chemotherapy, an experiment in carcinogenesis, and attempts to obtain tumors resistant to actinomycin D.

Materials

Actinomycin D stock solutions consisting of 100 μg./ml. were prepared by dissolving actinomycin D in 1 part ethanol diluted with 4 parts water. These solutions were sterilized and refrigerated. As required, all other solutions of actinomycin D were made by further dilution with saline. For the combination chemotherapy the water-soluble compounds were dissolved in saline; insoluble ones were suspended in 0.5 per cent carboxymethyl cellulose solution.

Mice used, all from Roswell Park Memorial Institute colonies, had an average weight of 20 gm. Sprague-Dawley rats, purchased elsewhere, had an average weight of 90 gm. The source and description of the tumors and the method of their propagation have been given previously (DiPaolo *et al.*, 1957).

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Methods and Results

Antitumor activity of actinomycin D. This has been tested on a variety of solid and ascites mouse tumors and on one rat tumor. In TABLE 1 are presented the pertinent results of intraperitoneal injections of 0.2 ml. of actinomycin D solutions given to mice twice daily, beginning the day after tumor transplantation and continuing for 7 or 14 days; rats were injected once daily for 14 days. Control animals were injected with saline. No significant weight losses were noted with the concentrations of actinomycin D used in these experiments.

Evaluations based on differences in the mean tumor diameter size, mean tumor weights, or mean survival time of 90 per cent of the control and treated animals reveal differences in the effectiveness of actinomycin D ranging from slight to complete inhibition. Comparison of results obtained with Ehrlich 2 ascites carcinoma and Krebs 2 ascites carcinoma demonstrates a preferential antitumor activity against the Krebs 2 ascites at a concentration as low as 30 μg./kg. body weight/day. The tumor inhibition obtained with S180 and lymphosarcoma 6C3HED ranges from 10 to 40 per cent using therapeutic doses; however, with a dosage of 100 μg./kg. for 7 days the S180 solid tumor was completely destroyed but the animals died by the eighth day. The ascites form of S180 obtained from our solid S180 was more sensitive to the carcinostatic action of actinomycin D than its respective solid tumor. No S180 ascites was found when the animals were treated intraperitoneally with 60 μg./kg. of body weight of actinomycin D. The antibiotic significantly extended the survival time of mice bearing the transplantable leukemia 1210. Actinomycin D caused up to 60 per cent growth inhibition of rat

TABLE 1
ANTITUMOR ACTIVITY OF ACTINOMYCIN D

Tumor	No. of animals	Deaths	Dose (μg./kg. body wt./day)	No. of I.P. injections*	Mean survival† 90% of animals (days)		Results†	Inhibition‡ (%)
					Expt.	Control		
Krebs 2 ascites	20	0	30	14	24	7	++	
Ehrlich 2 ascites	10	0	50	14	12	8	±	
Sarcoma 180	40	0	60	14			±	40
Sarcoma 180	10	10	100	14			−	
Sarcoma 180 ascites	10	0	60	14	50	15	++	
6C3HED	10	0	60	14			−	10
Leukemia 1210	20	0	60	14	30§	8	++	
C755	35	0	50	28			+	100
C_3HBA	15	0	30	28			+	94
Walker 256	10	0	15	14			±	62

* Animals were given injections twice daily 24 hours after tumor implantations; Walker 256 was injected once daily.

† For the ascites tumors, ± indicates 20 to 50 per cent increase in survival time of experimental group, and ++ indicates survival values exceeding 125 per cent.

‡ Per cent inhibition of S180 determined by mean diameter; of C755, C3HBA and Walker 256, by mean tumor weight. Inhibition was calculated 24 hours after the last injection.

§ Animals killed at 30 days.

carcinoma Walker 256. Inhibition of mammary gland tumors C3HBA and C755 was as great as 90 per cent on a wet weight basis using as little as 30 μg./kg. body weight. Complete inhibition of C755 occurred in several experiments with 50 μg./kg. body weight. Histological observations paralleled gross findings. In the case of mammary tumors, more mucus, fewer mitotic figures, and more vesiculation of the nuclei were apparent in the treated groups than in the control. The treated sarcomas were indistinguishable from the controls.

The responses of mouse adenocarcinoma C755 to actinomycin D were determined over a wide range because complete inhibition of this tumor had been observed using 50 μg. of actinomycin D/kg. of body weight. Tumor-bearing C57BL/6 mice were injected intraperitoneally twice daily for 14 days beginning 24 hours after tumor inoculation, and were killed 24 hours subsequent to the last injection. The results (FIGURE 1) show that one half the optimal dosages produced an inhibition of 69 to 89 per cent with an average inhibition of 79 per cent. Dosage of 10 to 12.5 μg. of actinomycin D/kg. of body weight produced 43 to 47 per cent inhibition while as little as 5 to 6.5 μg./kg. produced approximately 30 per cent inhibition of this sensitive tumor.

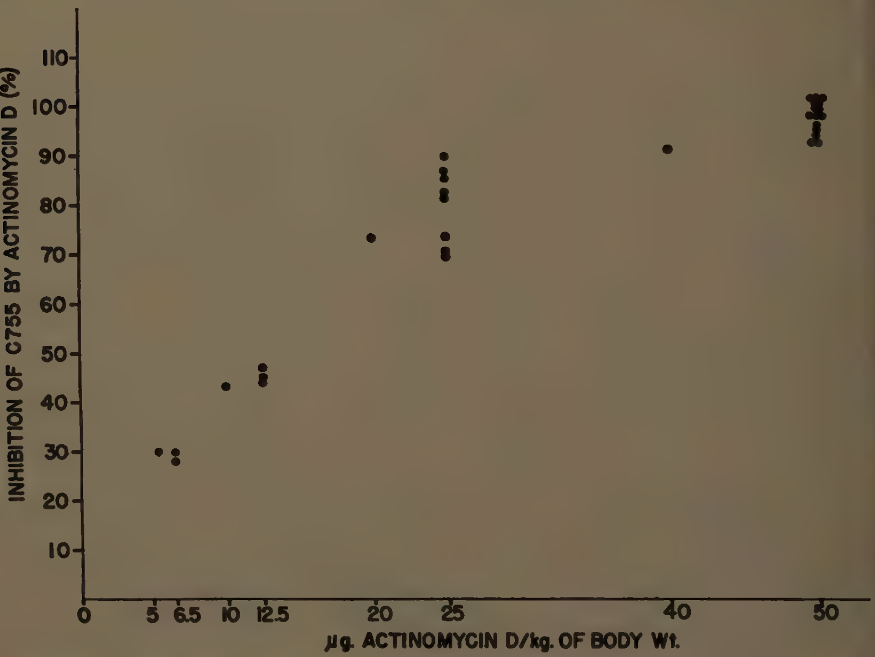

FIGURE 1. Per cent inhibition of a mouse adenocarcinoma resulting from a variety of concentrations of actinomycin D.

Studies in variation in the administration of actinomycin D have been previously reported (DiPaolo and Kolipinski, 1960). Single massive intraperitoneal injections have caused the complete disappearance of established solid lymphomas and Krebs 2 ascites tumor placed subcutaneously (TABLE 2). The injection of 700 to 1000 μg. of the drug/kg. body weight resulted in the complete reabsorption of established tumor. Second subcutaneous inoculations of Krebs 2 ascites into animals whose first tumors had been reabsorbed resulted in solid tumors that grew to 10 to 15 mm. in diameter and then were reabsorbed. A single intraperitoneal injection of actinomycin D was tried against the solid E2 strain of Ehrlich ascites. Swiss male mice were given subcutaneous injections of 0.2-ml. suspensions of 1:10 dilution of Ehrlich ascites cells with isotonic saline. Each animal was given 30 μg. of actinomycin D when its tumor was approximately 15 mm. in diameter. It was soon evident that no inhibition occurred in this case.

The solid Ehrlich 2 ascites tumor did not respond any better than its respective ascites form. Neither painting actinomycin D on the shaved back of Swiss mice nor oral intubation of a single massive dose of actinomycin D was effective upon mice given subcutaneous injections of 0.2 ml. (20 × 10^6 cells) of undiluted Krebs 2 ascites. Experiments carried out *in vitro* using fresh mouse skin showed that no dialysis of actinomycin D occurs through the skin over an 8-hour period. When 1500 μg. of actinomycin D were given orally to Swiss mice having established subcutaneous solid Krebs 2 tumors averaging 9 mm. the tumors grew to an average of 21 mm. in 16 days.

TABLE 2
EFFECTS OF SINGLE DOSES OF ACTINOMYCIN D ON GRAFTED TUMORS

Mouse strain	Tumor	Av. diameter of tumor at treatment (mm.)	I.P. dose (μg./kg. body wt.)	Deaths/total	Complete regression	Expt. duration (days)
DBA/1	Lymphoma solid	12	700	0/9	9	270
Swiss	Krebs 2 solid	10	875–1000	0/6	6	130
Swiss	Krebs 2 solid	13	1150–1200	43/97	37	70

Treatment of cells prior to injection. In another experiment utilizing Ehrlich 2 ascites cells, a suspension of cells was mixed with varying amounts of actinomycin D for 5 minutes, washed twice, and resuspended in normal saline prior to subcutaneous injection into Swiss mice. The use of actinomycin D within a concentration range of 3.5 to 50 μg./ml. resulted in measurable tumors as early as 13 days subsequent to inoculation with 5 million cells. No significant difference in tumor size was found between pretreated tumor cells and control group. Tumor growth was significantly depressed when cells in a concentration of either 50 or 60 μg. of drug/ml. of suspension was incubated for 10 min. at 37° C. prior to washing, resuspension in saline, and subcutaneous injection into mice. Growth was nonexistent or slight when compared to saline-incubated tumor cells; however, a loss of hair was noted at injection sites, indicating that not all the excess actinomycin had been removed by washing.

Study with phenoxazinones. The tumor-inhibiting properties of 5 compounds (2-amino-3*H*-phenoxazin-3-one, 1,9-diacetyl-2-hydroxy-3*H*-phenoxazin-3-one, 2-amino-3-oxo-3*H*-phenoxazin-1,9-dicarboxylic acid; 2-amino-1,4,6,9-tetramethyl-3*H*-phenoxazin-3-one, and 5*H*-benzo-*a*-phenoxazin-5-one) having structural features in common with actinomycin D were also investigated because of the potency of actinomycin D in destroying animal tumors. DiPaolo and Kolipinski (1960) have shown that actinomycin D and 2-amino-3*H*-phenoxazin-3-one and 2-amino-3-oxo-3*H*-phenoxazin-1,9-dicarboxylic acid of this group inhibited the growth of several species of *Clostridia*. These compounds were suspended in 0.5 per cent carboxymethyl cellulose in saline. Treatment and evaluation were identical to those used with actinomycin D. These compounds were well tolerated at 8 to 15 mg./kg./day. Using the limited available quantities of these compounds, no dramatic inhibition, as had been found with actinomycin D, was noted with any of the phenoxazinones in the amounts used (TABLE 3).

TABLE 3

EFFECT OF PHENOXAZINONES ON TUMOR-BEARING ANIMALS

Compound, tumor	No. of animals	Dose (mg./kg. body wt./day)	No. of I.P. injections*	Mean survival† 90% of animals (days)		Results†	Inhibition‡ (%)
				Expt.	Control		
2-Amino-3*H*-phenoxazin-3-one							
Krebs 2 ascites	10	8	14	12	13	—	
Ehrlich 2 ascites	10	15	14	25	15	±	
Sarcoma 180	10	10	14			—	−9
Carcinoma 755	20	8	28			—	1
2-Amino-1,4,6,9-tetramethyl-3*H*-phenoxazin-3-one							
Krebs 2 ascites	10	8	14	15.4	14.7	—	
Ehrlich 2 ascites	20	15	14	14	15	—	
Carcinoma 755	10	15	28			—	4
Sarcoma 180	10	8	14			—	1
1,9-Diacetyl-2-hydroxy-3*H*-phenoxazin-3-one							
Ehrlich 2	10	8	14	12.3	15.3	—	
Sarcoma 180	10	15	14			—	6
Carcinoma 755	10	8	28			—	10
2-Amino-oxo-3*H*-phenoxazin-1,9-dicarboxylic acid							
Ehrlich 2	10	15	14	15	13.8	—	
Carcinoma 755	10	15	28			—	4
5*H*-Benzo-*a*-phenoxazin-5-one							
Ehrlich 2	10	8	14	12.4	15.3	—	
Krebs 2	10	15	14	25.4	15.5	±	
Sarcoma 180	10	15	14			—	−1
Carcoma 755	10	8	28			—	11

* Animals were given injections twice daily 24 hours after tumor implantation.

† For the ascites tumors, ± indicates 20 to 50 per cent increase in survival in experimental group, and ++ indicates survival exceeding 125 per cent.

‡ Inhibition of S180 was determined by mean diameter; of C755 and C3HBA, by mean tumor weight. Inhibition was calculated 24 hours after the last injection.

Attempts to obtain a resistant tumor. Eventually all types of human cancer that respond to chemotherapy develop resistance to further treatment. Moore *et al.* (1958) noted that patients with and without temporary regression developed an accelerated metastatic growth rate subsequent to therapy with actinomycin D. Elucidation of the mechanism of this resistance would be valuable in learning how actinomycin D acts and in designing compounds that would be active against resistant cells.

Attempts have been made to change the actinomycin D-sensitive mouse adenocarcinoma C755 into a resistant tumor. C57BL/6 mice with the sensitive tumor were treated with 50 μg. of actinomycin D/kg. of body weight 5 times a week for 2 weeks, beginning 24 hours after tumor inoculation. Sixteen days subsequent to tumor inoculation, the tumors were transplanted again into untreated animals. The second, fourth, and sixth tumor generations were not treated. All subsequent generations of tumor-bearing mice were treated with 50 μg. of actinomycin D/kg. At irregular intervals these tumors were treated with either 50 μg. or 25 μg. of actinomycin D/kg. of body weight or with saline. The per cent resistance was determined by subtracting the per cent inhibition from 100 per cent. FIGURE 2 shows that, although at the 28th generation 50 per cent growth occurred with 50 μg. of actinomycin D/kg. of body weight, after 40 generations the same dosage still produced 55 per cent inhibition of C755, indicating that the tumor is adapted or resistant to the extent of 45 per cent in the presence of actinomycin D. The 25-μg. doses of actinomycin on these tumors resulted in inhibition that was less than half that obtained with the same dosage on completely sensitive tumors.

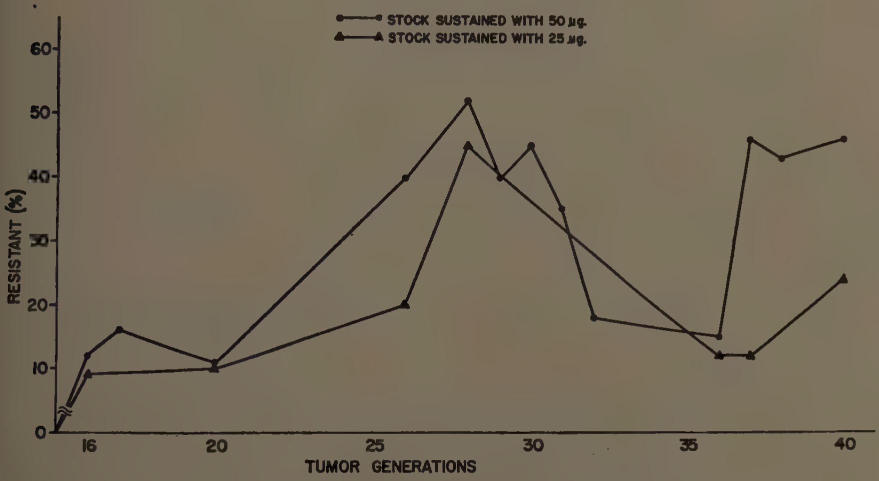

FIGURE 2. Tests for degrees of resistance of adenocarcinoma C755 to 50 μg. of actinomycin D/kg. of body weight by various tumor generations.

In a second experiment, tumor-bearing mice were treated with 12.5 μg. of actinomycin D/kg. of body weight for the first 3 tumor generations, followed by twice that amount through the 40th generation. This tumor in C57BL/6 mice was also challenged at irregular intervals with 50 μg. of actinomycin D/kg. of body weight or with saline. FIGURE 2 shows that this tumor is less resistant than the tumor that was treated regularly with the higher concentration of actinomycin D.

It is concluded that this sensitive tumor is not readily made resistant to actinomycin D.

Combination chemotherapy. Several drug combinations were studied in an effort to gain insight into the mechanism of action of actinomycin D that results in regression of mouse tumors. The sensitive mouse adenocarcinoma, C755 and the relatively insensitive mouse sarcoma S180 were used. Each combination reported was tried twice. Tumor-bearing mice received the calculated amount of materials once daily for 7 or 14 days, beginning 24 hours subsequent to tumor transplantations. In the case of Swiss mice bearing S180, the animals were weighed, and the average tumor diameter calculated 24 hours subsequent to the last injection, as well as 1 and 3 weeks later, in order to determine whether any tumor reabsorption or delayed toxicity was apparent. The inhibition of C755 was determined by comparing the average wet weights of tumors from treated and control groups.

The maximal amount of actinomycin D used against S180 was 60 μg./kg./day. Although ordinarily 50 μg./kg./day is used on C57BL/6 mice bearing C755, 25 μg./kg./day was used because the lower dose is still capable of producing an average of 79 per cent inhibition and facilitates evaluation of calculations comparing the combination of drugs to actinomycin alone. In most cases the higher concentration was incorporated into the various combination groups as a positive control.

The drugs studied in combination with actinomycin D include triethylene thiophosphoramide, 6-mercaptopurine, nitrogen mustard, Carzinophylin, urethan, 5-fluorodeoxyuridine, and A-methopterin; also studied were β-2-thienylalanine, methioprim, dihydrostreptomycin, oxythiamine, and sulfathiazole, which were found to be synergistic with actinomycin D in inhibiting *B. subtilis* 6051, and azaserine, which was antagonistic in combination with actinomycin D on *B. subtilis* (Slotnick, unpublished). For most drugs the highest dose used was that reported by Clarke (1955), Sugiura (1956), or Gellhorn *et al.* (1958). The highest doses of methioprim and oxythiamine were determined by Holland (1958). Where no information was readily available for one of the tumor systems, an effort was made to test the compound at a well-tolerated dose.

TABLE 4 presents the 13 drugs and the highest dosage concentration used in the combination studies with actinomycin D on S180 tumors; TABLE 5 gives the 11 drugs and the highest dosage used in the combination with actinomycin D on C755 tumors. The dosage plane (FIGURE 3) shows the various groups constituting each experiment. Actinomycin D is represented by the symbol X, while the second drug of each experiment is indicated by the symbol Y. In some experiments the combination of X + Y was omitted. This diagram permits both diagonal and rectangular comparisons. The interpretations presented are, for the most part, based on diagonal comparisons.

In this procedure the combination (X/2) + (Y/2) is subtracted from (X + Y)/2, and the combination (X/4) + (Y/4) is subtracted from [(X/2) + (Y/2)]/2. If the difference in either case results in a minus number, synergism is indicated, while a positive number reflects antagonism.

TABLE 4
DRUGS TESTED IN COMBINATION STUDIES WITH ACTINOMYCIN D ON SARCOMA 180

Code	Drug	High dose*
TSPA	Triethylene thiophosphoramide	4
6-MP	6-Mercaptopurine	50
ThAl	β-2-Thienylalanine	500
$CH_3$3-P	Methioprim	300
2H Strept	Dihydrostreptomycin	500
OxyTh	Oxythiamine	100
HN_2	Nitrogen mustard	0.5
CZ	Carzinophylin	2500†
AzS	Azaserine	4
U	Urethan	500
S	Sulfathiazole	500
5-FUDR	5-Fluorodeoxyuridine	50
Ameth	A-Methopterin	1.5

* Mg./kg. body weight/day.
† Units.

TABLE 5
DRUGS TESTED IN COMBINATION STUDIES WITH ACTINOMYCIN D ON C755

Code	Drug	High dose*
TSPA	Triethylene thiophosphoramide	4
ThAl	β-2-Thienylalanine	250
CH_3S-P	Methioprim	300
2H Strept	Dihydrostreptomycin	400
OxyTh	Oxythiamine	100
HN_2	Nitrogen mustard	0.6
CZ	Carzinophylin	2000†
AzS	Azaserine	3
U	Urethan	250
5-FUDR	5-Fluorodeoxyuridine	50
Ameth	A-Methopterin	1.5

* Mg./kg. body wt./day.
† Units.

The differences obtained were then subjected to a *t* test to determine whether they were significant at the 5 per cent level of confidence. Such analysis showed that no drug combination is statistically significant in synergism or antagonism against S180 even though differences in inhibition are found when Carzinophylin or azaserine are combined with actinomycin D (TABLE 6). Rectangular comparison of inhibition by the high doses revealed that actinomycin D inhibition may be additive when combined with Carzinophylin since inhibition obtained with the one-half dose combination exceeded that obtained with either drug alone.

Summary of results of combination chemotherapy on growth of C755 is in TABLE 7. Diagonal analysis of the combination tried against this tumor showed that azaserine was antagonistic in combination with actinomycin D. Slotnick, using *B. subtilis*, found similar results.

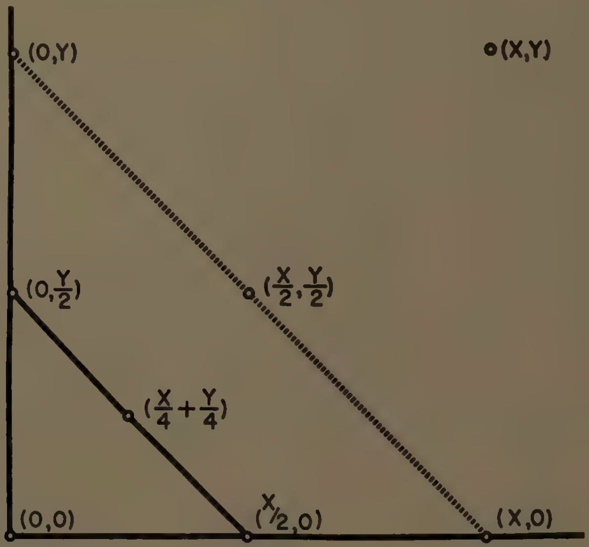

FIGURE 3. The various groups constituting most of the combination chemotherapy experiments are indicated by X (actinomycin D) and Y (the second drug).

TABLE 6
RESULTS OF COMBINATION CHEMOTHERAPY ON GROWTH OF SARCOMA 180

Treatment	Drugs tested*												
	TSPA	6-MP	ThAl	CH_3S-P	2H Strept	OxyTh	HN_2	CZ	AzS	U	S	5-FUDR	Ameth
Actino. D 60 μg./kg., X	23	37	35	42	27	27	37	27	26	21	27	26	42
Actino. D 30 μg./kg., X/2	16	22	20	20	17	17	25	16	16	15	17	16	20
Test agent high, Y	33	20	25	15	26	25	18	9	41	15	19	47	28
Test agent low, Y/2	2	16	20	3	17	18	18	8	38	17	22	44	17
X + Y	25	45	29	25	8.3	26	40	45			23		
(X/2) + (Y/2)	19	32	30	20	17	17	37	31	31	20	19	25	35
(X/4) + (Y/4)	13	23	18	10	18	17	19	10	28	1	18	24	16

* Per cent inhibition of tumor growth. For code to abbreviations see TABLES 4 and 5.

Carcinogenesis experiment. A one-month-old male and a female Swiss mouse of ICR/Ha strain and a two-month-old male and a female DBA/1 mouse were used. Four groups of Swiss mice and 4 groups of DBA/1 mice, each consisting of 8 mice, were injected twice weekly for 4 months either intraperitoneally or subcutaneously (in different sites) with 200 μg. of actinomycin

D/kg. of body weight. Preliminary experiments had proved that this amount of actinomycin D over several weeks did not result in significant loss of body weight or in death of the animals. The injections were continued for 4 months; animals were weighed twice weekly for 8 months. The results in TABLE 8 are from autopsies performed on animals that survived to 20 to 21 months of age. It should be pointed out that none of the tumor-bearing animals was tested for polyoma antibodies. The breast tumors were the most numerous; 9 were found in Swiss mice and DBA/1 mice, both treated intraperitoneally and subcutaneously. One was in a Swiss male that had been treated intraperitoneally. Of the total 21 tumors found, 14 occurred in Swiss mice. The incidence of lung tumors is probably within the expected spontaneous rate. Hepatomas were found in 2 Swiss males and a DBA/1 female, all of which had been treated with subcutaneous injections.

TABLE 7
RESULTS OF COMBINATION CHEMOTHERAPY ON GROWTH OF C755

Treatment	Drugs tested*										
	TSPA	ThAl	CH_3S-P	2H Strept	OxyTh	HN_2	CZ	AzS	U	5-FUDR	Ameth
Actino. D 25 μg./kg., X	85	65	73	73	66	66	66	66	73	73	66
Actino. D 12.5 μg./kg., X/2	47	45	45	45	47	47	45	44	45	45	47
Test agent high, Y	95	20	17	0	20	72	60	85	62	53	21
Test agent low, Y/2	84	0	22	20	6	28	−16	54	36	54	−67
X + Y	100		78	61		84		90	90		
(X/2) + (Y/2)	100	62	57	44	33	63	50	40	61	80	76
(X/4) + (Y/4)	88	22	36	27	29	48	32	36	50	40	−5

* Per cent inhibition of tumor growth. For code to abbreviations see TABLES 4 and 5.

TABLE 8
SUMMARY OF DATA FROM CARCINOGENESIS EXPERIMENT USING ACTINOMYCIN D

Mice (no. and kind)	Sex	Mode of injection	Survivors	Tumors					Ovarian cyst
				Lung*	Liver	Breast	Skin†	Spleen	
8 Swiss	F	I.P.	7	2		3			
8 Swiss	F	S.C.	7	1		1	1	1‡	2
8 Swiss	M	I.P.	6	1	1	1			
8 Swiss	M	S.C.	8		1		1		
8 DBA/1	F	I.P.	8	1		2			1
8 DBA/1	F	S.C.	8		1	2			
8 DBA/1	M	I.P.	7						
8 DBA/1	M	S.C.	8				1		

* Adenoma.
† Squamous cell carcinoma.
‡ Hemangioma.

When actinomycin D was injected subcutaneously, 2 skin squamous cell carcinomas and 1 adenoacanthoma (squamous cell carcinoma with glandular elements, FIGURE 4) were found among the Swiss and DBA/1 animals. The most striking aspect of this experiment, initiated with 64 mice and terminated with 61, is the skin tumors resulting from subcutaneous injection of actinomycin D. The number of tumors is large in relation to the number of animals treated subcutaneously; moreover, I have never seen spontaneous squamous cell carcinoma of the skin in untreated animals. Recently, Kawa-

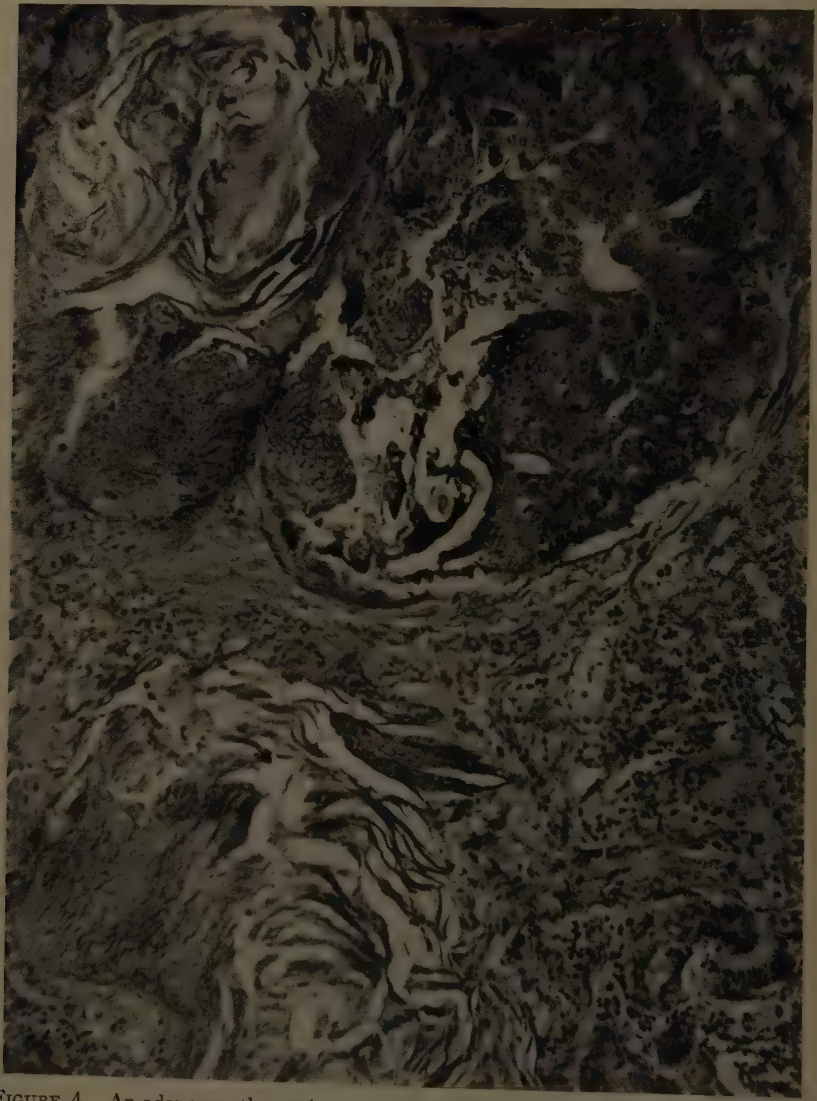

FIGURE 4. An adenoacanthoma (squamous cell carcinoma with glandular elements) from a DBA/1 mouse injected subcutaneously with actinomycin D.

mata *et al.* (1958) have also found that actinomycin 1048A causes sarcomas when repeatedly injected subcutaneously into the right dorsal side of mice. Most of the tumors in this experiment, except the skin tumors, are probably within the expected spontaneous range. It would be interesting to repeat and enlarge that part of the experiment which gave rise to skin tumors.

The most common nonmalignant change found in these animals was the number of organs that had reactive changes consisting of moderate lymphoid hyperplasia. These cells were found in the kidneys of mice belonging to all groups injected, as well as in the lungs and livers in some groups. Lymphoid hyperplasia of the spleen was found in four cases. Some animals from all groups exhibited varying amounts of focal necrosis and fatty degeneration of liver.

Summary and Conclusion

Actinomycin D is an extremely potent inhibitor; a few micrograms per kilogram of body weight inhibit most transplantable mouse and rat tumors tested. A relatively complete molecule of actinomycin D including the amino acid side chains is required for tumor inhibition, since the chromophore group alone and other related phenoxazinones were ineffective.

Attempts to convert a sensitive tumor to an actinomycin D-resistant tumor have been unsuccessful.

Although there is only incomplete knowledge of the mode of action of actinomycin D or of the other compounds, combination therapy was attempted for the purpose of obtaining information preliminary to further study. Therapeutic effectiveness was not increased when actinomycin D was used in combination with other drugs. When a deoxyribonucleic acid-blocking agent, 5-fluorodeoxyuridine, was combined with actinomycin D, presumably an RNA-blocking agent, no increased tumor inhibition was noted. Combination with azaserine was antagonistic to actinomycin D tested on adenocarcinoma C755.

The carcinogenicity of actinomycin D is suggested by the occurrence of the 3 squamous cell carcinomas in 31 mice injected subcutaneously and in none of 40 controls.

The major problem with actinomycin D is its inherent toxicity. Further study may reveal, without loss of the tumor-inhibiting properties of actinomycin D, a derivative with less toxicity or a combination resulting in decreased toxicity.

Acknowledgments

The author is indebted to Merck and Company, Inc., for supplying the actinomycin D; to E. W. Malmberg, Ohio State University, for the phenoxazines, except the 5-*H*-benzo-*a*-phenoxazin-5-one, and to I. Bross and J. Dowd of the Department of Statistics for stimulating discussion concerning combination therapy. The technical assistance of Mary Black, Nick Skalski, and George Olshevsky is gratefully acknowledged.

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Part III. Clinical Significance

CLINICAL STUDIES OF ACTINOMYCIN D WITH SPECIAL REFERENCE TO WILMS' TUMOR IN CHILDREN*

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This publication commemorates the 20th anniversary of the discovery of actinomycins by Selman A. Waksman. The vast importance of products of the actinomyces in the treatment of infectious disease is now a part of medical history. Twelve years after the isolation of actinomycin by Waksman and Woodruff in 1940,[1] Hackmann[2] in 1952 demonstrated the carcinolytic effect of actinomycin C. Studies made by Ravina and others in 1954[3] pointed clearly to the possible usefulness of this substance in the treatment of some forms of cancer in man, such as Hodgkin's disease. The studies summarized in this paper had their origin in discussions with Waksman in 1954. The antibiotic selected for initial study, actinomycin D, very quickly proved to be, on the basis of weight, the most powerful anticancer agent against transplanted tumors in the mouse that we had studied up to that time.[4,5] Clinical studies, begun as soon as extensive toxicological studies were completed, demonstrated no value in the treatment of acute leukemia in children. When administered to children with advanced Wilms' tumor, rhabdomyosarcoma, Ewing's tumor, and Hodgkin's disease, however, there was sufficient evidence of clinical improvement to warrant trials against many different kinds of cancer in man.[6]

Our clinical studies, conducted over the past five years, include observations of 330 patients having many different forms of disseminated or advanced cancer. Of these, 275 patients were children and 55 were adults. These studies form the background for the opinions expressed here, although discussion will be limited to observations made on children with Wilms' tumor of the kidney.

Actinomycin D was injected intravenously, with special precautions taken. Since local necrosis is caused, if extravasation occurs, it was never administered directly with syringe and needle. It is administered best into the side arm as an intravenous infusion or by means of a commercially available scalp-vein set. In our earlier studies, the dose administered was 75 μg./kg. for a total course when combined with X-ray therapy, and 100 μg./kg. per course when given alone. During the past two years we have employed a somewhat lower dose, which has been better tolerated, and are now using 60 μg./kg. combined with X-ray therapy or 75 μg./kg. alone. The dose schedules should be selected for the individual patient, and treatment should be stopped if any signs of important degrees of toxicity are recognized. Single injections vary from 100 to 400 μg., and the course usually lasts seven to fourteen days. Actinomycin D causes depression of the bone marrow, thrombocytopenia being the first indication of toxicity. Therapy is interrupted if the level of platelets falls below 150,000/cu. mm. Evidences of toxicity, such as stomatitis and gastrointestinal

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alterations, are rarely seen at the lower dose range. Alopecia, originally found in about one half of the patients, is now rare in our experience.

Wilms' tumor of the kidney is one of the most common solid tumors in infants and children. The gross and histological pictures vary greatly, but all forms of Wilms' tumor must be regarded as malignant. In our experience of the last 30 years, the cure rate for all infants and children with Wilms' tumor has varied from 40 to 48 per cent, when surgery alone, or surgery and X-ray treatment to the kidney region after surgery, were the methods employed. Under the same forms of treatment about 80 per cent of children under one year of age, with Wilms' tumor, have been cured. Metastases occur most frequently in the regional lymph nodes and the lungs and, less commonly, in the brain, the liver, and the bones. The tumor is sensitive to X-ray therapy. When striated muscle is an important component of the tumor, radiotherapy is less effective. Metastases in the lungs do respond to X-ray treatment, but the dangers of radiation pneumonitis and of damage to bone growth prevent the employment of the full therapeutic value of this form of treatment. Metastases in the lung are usually multiple; in rare instances they are solitary. When metastases are multiple, exitus usually occurs in four to twelve months.

Treatment of Wilms' Tumor

Early in our studies we witnessed the diminution of metastases of Wilms' tumor following the administration of actinomycin D alone. This observation has been abundantly confirmed.[7-9]

Our interest in the synergistic as well as potentiating action of actinomycin D on X-ray therapy was aroused by the appearance of erythema on the skin of a child who had received X-ray treatment directly to a pulmonary metastasis, in an amount which, by itself, would not have caused such a skin reaction. Inquiry revealed that the child had received actinomycin D five months earlier. Careful observations of the effect of X-ray treatment and actinomycin D on children with metastatic cancer, and further laboratory observations of mice, revealed a degree of synergism that amounts to a potentiation of radiotherapy by actinomycin D, of importance to the patient.[10,11] Most of our experience was obtained with the X rays employed under the following technical conditions: kilovolts, 250; added filter, 0.4 mm. Cu, 0.25 mm. Cu, 1 mm. Al (Thoraeus type); half-value layer, 2.7 mm. Cu; milliamperes, 15; and target–skin distance, 50 cm.

Radiotherapy to the primary site of the tumor, after its surgical removal, was given usually in the amount of 3000 r in 20 treatment days over a period of three and one-half weeks. Pulmonary metastases were treated with a total dose of, usually, 1200 r in 10 treatment days over a period of one and one-half weeks. Somewhat larger doses, from 2000 r to 3000 r, given over two and one-half to three and one-half weeks, were employed for the treatment of metastases in bone, brain, and liver.

In the five-year period from 1955 to 1960, 37 children with widespread metastases in the lungs and advanced cancer were seen. The disease was regarded as advanced when more than two lobes of the lungs were involved or when metastases had been found in more than two organ systems. Of 29 patients who received actinomycin D intravenously and concomitant X-ray treatment, 10

(35 per cent) are still living, with no evidence of cancer demonstrated by X ray, 10 to 35 months after onset of symptoms, and 4 to 33 months after the last evidence of metastasis disappeared under treatment. Six of these 10 children have survived for more than 26 months after the first evidence of the tumor. There were 8 children who came to our attention in states of advanced cancer with widespread metastases; they had not been treated with the combination of X ray and actinomycin D. All 8 have died.

A detailed study of the patients with Wilms' tumor cared for during two years beginning January 1, 1956 permits another form of analysis. The patients will be divided into three groups. The first group of children came to our care with late advanced cancer. They had been treated by surgical removal of the primary tumor and X-ray therapy to the kidney region after operation. Metastases developed later. At that time actinomycin D and X-ray therapy were applied in combination. Of 8 children in this group, 6 lived for 6 to 24 months; 2 are living and show no evidence of disease, one 32 months and one 50 months after discovery of the tumor. A second group, with advanced cancer when they came to our attention, consisted of two patients. Only surgery originally had been employed. Both patients died, one 12 months and the other 18 months after surgical removal of the primary tumor. A third group of 8 patients received treatment by the techniques of surgery and X ray in addition to actinomycin D from the time of the primary operation. One of these, a child with bilateral Wilms' tumors, died six months after discovery of the tumors. The remaining 7, treated with surgical removal of the primary tumor, X-ray therapy to the kidney region after operation, and actinomycin D from the time of the operation, are living, with no detectable evidence of tumor, 32 to 38 months after removal of the primary mass.

Discussion

Actinomycin D is a valuable adjunct to the treatment of Wilms' tumor in infants and children. It is a toxic material, but serious toxicity may be prevented by careful calculation of the dose for a given patient. The toxicity is a serious drawback to the exploitation of the full carcinolytic properties of actinomycin D. It is hoped that the brilliant chemical studies of such men as Brockmann and Johnson, whose contributions appear elsewhere in this monograph, will make possible the synthesis of related materials that, it may be hoped, will retain the anticancer potency of the antibiotic without its toxic component. Until the factor of toxicity is solved, either in such a manner or by biological studies, this remarkable chemical substance will have limited value as an anticancer agent in man.

The potentiation of X-ray treatment effects by actinomycin D opens an era of great importance in the treatment of cancer. Moreover, the effectiveness of X-ray treatment may be increased greatly when used in combination with substances such as actinomycin D. Of particular importance is the possibility of destroying pulmonary metastases, by treatment so combined that X-ray therapy will be effective at a level lower than the minimum required to produce radiation pneumonitis. Thorough exploration of this combined treatment of tumors regarded as radio-insensitive should be productive of important observations on the response of cancer in man.

Actinomycin D is carcinolytic when used alone in the treatment of children with widespread metastases from Wilms' tumor. It is much more effective when used in combination with X-ray therapy. This combination is the best method yet discovered for the treatment of metastases from Wilms' tumor. A long-range program designed to prevent metastasis from Wilms' tumor involves the administration of actinomycin D at the time of surgical removal of the primary tumor, and local radiotherapy for all patients with Wilms' tumor, including those in whom no evidence of metastasis can be demonstrated. The addition, however, of a potentially toxic agent in the treatment of a child with Wilms' tumor who may, on a statistical basis, have a 40 to 50 per cent chance of complete cure from surgery and postoperative radiation only, calls for caution and skill. The addition of this form of chemotherapy to the techniques of surgery and radiotherapy in the treatment of a child with Wilms' tumor is directed toward the 50 or 60 per cent of such children who have not been cured by former methods of treatment. On the basis of our experience of the past five years it may be anticipated that the mortality rate will decrease markedly if this regimen is followed from the time of the discovery of a Wilms' tumor in an infant or child.

It may be anticipated, too, that the research of S. Waksman, opening the door to the actinomycins, will lead to a rewarding yield of antibiotics of carcinolytic importance to man.

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Discussion of the Paper

M. C. Li (*Sloan-Kettering Institute for Cancer Research, Memorial Center for Cancer and Allied Diseases, New York, N. Y.*): I am very much interested in the combination of actinomycin D and radiotherapy in the treatment of metastatic Wilms' tumor, which S. Farber and his co-workers have described. During the last two and one-half years, we have been using an alkylating agent, in place of radiotherapy, an antimetabolite, and actinomycin D for the treatment of patients with advanced cancer of the testis. Of the 23 patients so treated, seven showed a complete or nearly complete resolution of pulmonary metastases and of palpable masses. Four of the seven have continued in good health for eight to eighteen months or more, and 3 had relapse of disease following a complete remission. In cases of choriocarcinoma the response was associated with a fall of chorionic gonadotropin titer to negative values. Five of the 23 patients showed a partial regression of metastases, and four of these have died in spite of continued therapy.

Wilms' tumor, cancer of the testis, and choriocarcinoma of the uterus are generally considered embryomas. Unlike the patients with uterine choriocarcinomas, most of the males having metastatic choriocarcinoma with or without embryonal carcinoma, teratocarcinoma, or both have not responded satisfactorily to antimetabolite therapy given alone.[1,2] Nine and eight other patients having advanced cancer of the testis were treated either with an alkylating agent plus actinomycin D or with 6-mercaptopurine plus 6-diazo-5-oxo-L-norleucine combination, respectively. Only two treated with the former and one treated with the latter responded favorably. The original observations have been published elsewhere.[3,3a,4] Our conclusion is in agreement with Farber's, namely, that actinomycin D may have augmented the effect of radiomimetic agents or vice versa in the last group of cancers.

We have obtained another interesting result from the treatment of a woman having choriocarcinoma of the uterus with metastases to the lungs. She was initially treated with A-Methopterin and responded only partially. Continued A-Methopterin therapy did not bring about further regression of pulmonary metastases. She was then treated with actinomycin D and chlorambucil in combination. There was a complete resolution of the pulmonary metastases associated with a fall of urinary chorionic gonadotropin to negative values. This woman has been in good health and has had no evidence of disease for the last 15 months since the initial combination therapy.

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CLINICAL EXPERIENCES WITH ACTINOMYCINS D, KS2, AND F_1 (KS4)*

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This report is an evaluation of actinomycins D, KS2, and F_1, based on the treatment of 363 cases of metastatic cancer in children and adults. Actinomycin D[1] consists of a chromophor group and 2-methylvaline, sarcosine, L-proline, D-valine, and threonine in the two polypeptide chains. Actinomycin KS2 is considered to be identical with actinomycin C and consists of several actinomycins. Actinomycin F_1,[2] also known as actinomycin KS4, a biosynthetic actinomycin, differs in structure from actinomycin D in the presence of alloisoleucine instead of proline.

Sugiura and Schmid[3] (and Sugiura in a personal communication) have reported that both actinomycin D and F_1 had a marked inhibitory effect on Ridgway osteogenic sarcoma in mice at a dosage of 0.025 mg./kg. × 7 and 0.5 mg./kg. × 7, respectively. Clarke (personal communication) found that both actinomycin D and actinomycin F_1 at tolerated doses were ineffective in sarcoma 180 and adenocarcinoma 755. At toxic doses, both compounds had slight inhibitory effect in these two tumors. Actinomycin F_1 inhibited tumor growth in a spontaneous mammary tumor in mice at tolerated nontoxic doses, whereas actinomycin D had no inhibitory effect on this tumor even when given at toxic doses. Because of this inhibitory effect of actinomycin F_1 in contrast to actinomycin D on a spontaneous mammary tumor, the clinical effects of actinomycin F_1 were compared with those of actinomycin D.

Materials and Methods

Actinomycin D was supplied in a solution containing 500 γ/ml. and in 1 mg. oral tablets.

Actinomycin KS2 was supplied as 0.5 mg. oral capsules.

Actinomycin F_1 was available in powder form in ampules, each containing 2.5 mg. The powder was readily soluble and dissolved in normal saline to a concentration of 1 mg./ml. for intravenous use. Each vial was further diluted with normal saline so that the volume injected was approximately 20 ml. Care was exercised to avoid extravasation of the drug, since this causes severe cellulitis.

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Results

Actinomycin D. In children the effect of actinomycin D on cancer has been previously reported.[4]

Dosage: in children, a single course of intravenous administration of actinomycin D consisted of 15 μg./kg. of body weight per day for five doses, or a total of 75 μg./kg. If after two weeks there were no signs of toxicity and no therapeutic effects were obtained, the same dosage was repeated. Some patients have received two to five or more separate courses over a period of several months.

Table 1

Number and Diagnosis of Children Showing Objective Response to Actinomycin D Alone and Actinomycin D Plus X-Ray Therapy

	Total	Actinomycin D			
		Alone		+ X ray	
		Suitable for evaluation	Objective response	Suitable for evaluation	Objective response
Wilms' tumor	46	20	7	19	14
Neuroblastoma	25	10	4	13	7
Rhabdomyosarcoma	15	6	1	10	6
Bone sarcoma	18	3	0	10	2
Sarcoma botryoides	5	2	1	4	4
Embryonal carcinoma	4	1	1	1	0
Hodgkin's disease	5	4	2	1	0
Reticulum cell sarcoma	5	2	1		
Lymphosarcoma	4	2	2		
Acute leukemia	2	2	0		
Chronic leukemia	1	1	0		
Teratoma	2	1	0	1	1
Miscellaneous	7	5	0	1	0
Totals	139	59	19	60	34

The oral dosage of actinomycin D was 3 to 10 mg. daily in three divided doses.

Table 1 summarizes the data concerning the type of neoplastic disease, number of patients studied, and the objective response of the disease in children treated with intravenous actinomycin D alone and with actinomycin D and X-ray therapy.

For various reasons, not all the patients started on actinomycin D received an adequate trial of therapy, and these were not included in the evaluation of the therapeutic results.

These objective responses represented a decrease in size of the primary tumor and/or metastases by actual measurements or roentgenograms and lasted for a period of at least four weeks. The first group of patients had either not received X-ray therapy or had been irradiated at least two months before receiving actinomycin D. Therefore the objective response shown was considered to be due to actinomycin D alone. The second group received actinomycin D either simultaneously or within a period of one to two weeks before or after X-ray therapy.

Fifty-nine children with metastatic cancer were treated with actinomycin D alone and were suitable for evaluation (TABLE 1). A total of 19 of the 59, or 32 per cent, of the children showed definite objective anticancer effects to actinomycin D alone. Of these, only the children with metastatic Wilms' tumor have shown substantial clinical benefit.

One child had the disease at two years of age with pulmonary metastases, and is now six years of age and has no evidence of disease. Another child, who had pulmonary metastases at the onset at the age of three years, survived 27 months. One had no recurrence for seven months; the other two showed remarkable regression of pulmonary metastases for 1½ to four months.

Sixty children were treated with approximately 50 per cent of the estimated therapeutic dose of X rays and one or more courses of actinomycin D intravenously. While the data in TABLE 1 suggest that the effects of X rays are enhanced by actinomycin D, there are no control data available on the effects of X-ray therapy alone.

Forty-eight patients received actinomycin D orally to the point of toxicity. Of these, six received actinomycin D only by the oral route and 42 also received it intravenously, but at a different time. Of these 48 patients, the only response was seen in a child with reticulum cell sarcoma, who had a decrease in the size of metastatic skin nodules while he received actinomycin D orally in a total dosage of 16 mg./kg. in 13 weeks. Because of persistent gastrointestinal symptoms, the oral dose was interrupted for two weeks and the nodules recurred. A second response was not obtained.

Oral administration of actinomycin D causes a more severe local effect on the digestive tract than does parenteral administration. This usually prevented the administration of equivalent amounts of the drug.

Forty-eight adult patients with 14 different tumor diagnoses were treated with intravenous actinomycin D alone. Forty-two of these patients were considered to have been adequately treated. The criteria for adequacy of treatment and the degrees of toxicity produced were the same in this group as in the actinomycin F_1-treated series. Nausea, vomiting, anorexia, and oral ulcerations were seen frequently, but thrombocytopenia and/or leucopenia were usually the dose-limiting factors.

The total dose achieved with actinomycin D was rarely more than 0.060 mg./kg., and was usually given daily in equally divided doses for five or six days. This is in contrast to the dose of 0.075 mg./kg. given to children here and to adults elsewhere,[5] in whom somewhat better results were observed.

There were no beneficial effects noted in this group of 48 patients, which included 7 cases of breast carcinoma, 8 cases of soft tissue sarcoma, and 27 other cases with 12 different diagnoses that were adequately treated. It must be noted that in a few cases beneficial results were seen in patients treated with actinomycin D and X ray simultaneously, but the evidence is not adequate to say at this time what the actinomycin D contributed to these results.

Actinomycin D has also been used in combination with other chemotherapeutic agents; therapeutic effects, notably in carcinomas of the testicle, have been seen.[6]

Actinomycin KS2. Actinomycin KS2 is also known as actinomycin C. This was given orally to 17 children. Of the 11 who received adequate dosage, seven had Wilms' tumor, two were patients with neuroblastoma, one with acute leukemia, and one with rhabdomyosarcoma.

Dosage: actinomycin KS2 was given orally at a daily dosage of from 3 to 6 mg.

Toxicity: the toxicity of actinomycin KS2 is similar to oral actinomycin D. Nausea and vomiting were observed in all patients. Occasionally systemic toxicity with alopecia and thrombocytopenia were seen. The severe nausea and vomiting resulted in weight loss, the limiting factor in continuing the drug for a longer period of time. Only Case 1 of these 11 children showed an objective response. A child with Wilms' tumor (Case 2) responded to actinomycin F_1, and was then put on maintenance therapy with oral actinomycin KS2, 2.0 to 4.5 mg. daily for 9 months, during which time he had no recurrence of pulmonary metastases. It is hard to determine whether actinomycin KS2 had any effect in prolonging the regression in this patient. The other six children had progression of the disease while receiving actinomycin KS2 orally. Then either intravenous actinomycin D or F_1 was given parenterally, with a satisfactory response in some cases.

Case 1: F.B., a 4½-year-old boy, diagnosed as having acute leukemia in July, 1958, was given 6-mercaptopurine from July 30 to October 9, and had a partial remission for six weeks with the stem cells in the bone marrow decreased from 100 per cent to 60 per cent. From October 9 to December 29 he was given Methotrexate, with very little hematological response. From January 4 to February 1, 1959, he was given Medrol, followed by a complete bone marrow remission that only lasted for two weeks. On February 18 the bone marrow aspiration showed 95 per cent stem cells, and his white count was 30,000. From February 18 to February 23 he was given actinomycin KS2 orally 2 to 4 mg. daily, to a total dose of 111 mg., or 5.7 mg./kg. The drug was discontinued because of nausea, vomiting, weight loss, mouth ulcers, and alopecia. The toxicity lasted for about one week. The bone marrow aspiration on April 1 showed 2 per cent stem cells, 14 per cent erythroids, 12 per cent lymphocytes, and 66 per cent myeloid cells. The bilateral cervical nodes, 2 to 3 cm., disappeared almost completely. The spleen decreased from 6 to 1 cm. and the liver from 8 to 3 cm. below the costal margins. From April 7 to May 25 actinomycin KS2, 2 to 6 mg. daily to a total dose of 149 mg., or 7.3 mg./kg., was given again to toxicity. Following the first course of actinomycin KS2, he had a clinical and hematological remission that lasted for seven weeks. On May 20, while he was receiving actinomycin KS2, the bone marrow aspiration showed 90 per cent stem cells. The dose was then increased to 6 mg. daily, rapidly leading to toxicity. On this dose he had a partial remission lasting for three weeks. He failed to respond to a third course of actinomycin KS2 or to other subsequent therapy, and expired in August 1959.

Comment: this patient with acute leukemia responded poorly to 6-mercaptopurine or Methotrexate. Only Medrol had produced a 2-week bone marrow remission. On oral actinomycin KS2, two brief remissions were obtained.

Actinomycin F_1. The major portion of this report is concerned with our experiences with actinomycin F_1.

Dosage in children: the average intravenous dose for a child less than three years of age was 2.5 mg./day and for a child between three and ten the daily dose was 5 mg./day. In some older children above ten years of age, a dose of 10 mg./day was tolerated. The total dosage varied from 12 mg. to 170 mg., or 0.9 to 7.5 mg./kg. in a period of 5 to 110 days. Excluding two extremes of variation, the average total dose in children was 1.5 mg./kg. (that is, 0.15 mg./kg. daily for 10 days).

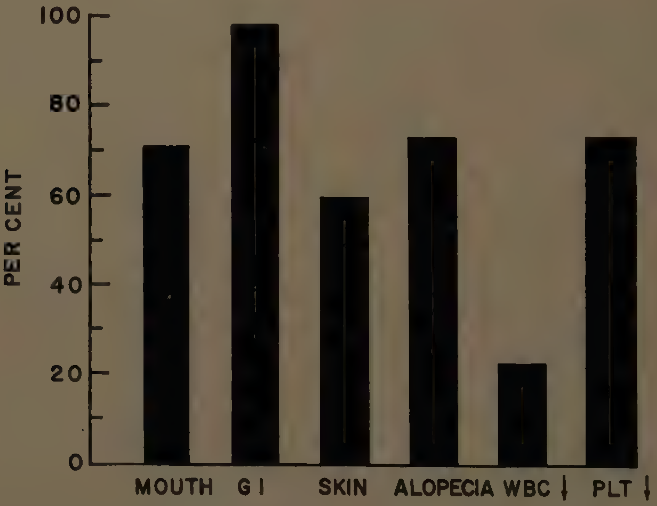

FIGURE 1. Percentage of children showing various toxic manifestations after intravenous administration of actinomycin F_1.

Dosage in adults: in adults the usual total dosage of actinomycin F_1 ranged from 0.6 to 1.0 mg./kg. given in a period ranging from 5 to 28 days.

During their therapy, patients were examined daily for signs of toxicity and evidence of therapeutic response. The concentration of hemoglobin and the leukocyte count were determined daily and the platelet counts twice weekly (daily in adults). Concentration of urea nitrogen in blood and uric acid in serum were determined at intervals of one to two weeks, and in some patients aspirations of sternal marrow and other chemical examinations of the blood were performed as indicated.

Only those patients who received an adequate trial of therapy (that is, in whom there was some form of systemic toxicity besides the nausea and vomiting) were included in the evaluation of the therapeutic results.

Toxicity in children: the toxic effects of actinomycin F_1 (FIGURE 1) were similar to that of actinomycin D:

(1) Redness and ulceration of the tongue and buccal mucosa usually de-

veloped within one to three days after the last dose of therapy and lasted for three to five days. These lesions were usually quite painful and were accompanied by an increase in salivation.

(2) Gastrointestinal disturbances: vomiting usually occurred two to four hours after each injection, and nausea, anorexia, and occasionally abdominal pain or diarrhea were noted during the course of therapy.

(3) There were skin reactions over previously irradiated areas, consisting of erythema and occasionally vesiculation and exfoliation. When actinomycin F_1 was given simultaneously with X-ray therapy, the skin reaction was greater than that expected with X-ray therapy alone.

Of the 30 children who received irradiation before the administration of actinomycin F_1 (FIGURE 2), 17 had exacerbation of erythema of the skin. This occurred when the actinomycin was given one, two, three, and four months and—in four patients—between six and 11 months after irradiation.

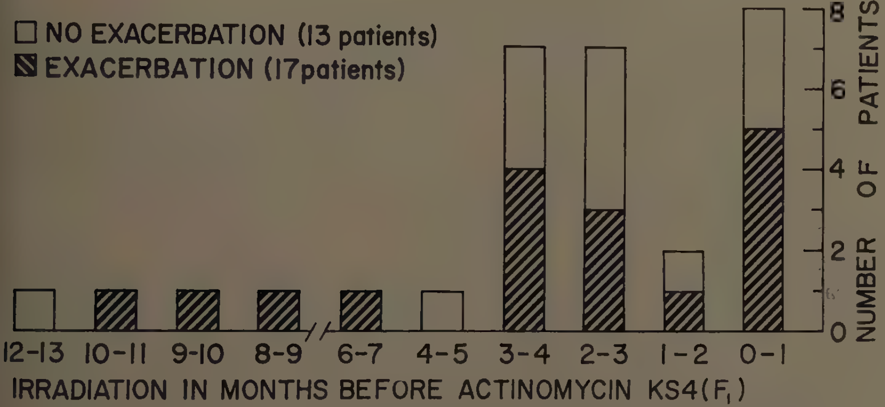

FIGURE 2. Number of children showing exacerbation of skin reaction by actinomycin F_1. This occurred when irradiation was given one to four months and—in four children—six to ten months before the administration of actinomycin F_1.

Five children and two adults developed allergic skin reactions with generalized erythema, and one child had urticaria after actinomycin F_1 therapy. Occasionally, acneiform eruption was observed in both adolescents and adults.

(4) Alopecia of varying severity developed one to two weeks after the completion of the course. This continued for two to four weeks and then regrowth occurred.

(5) Depression of the leukocyte count was usually temporary and consisted of a decrease from normal to below 3000/cu. mm. In some patients there was a neutropenia, with a decrease of the percentage of neutrophils from 60 to 80 per cent to 30 or 40 per cent. Occasional eosinophilia of 5 to 10 per cent or more was observed.

(6) Decrease of platelet count from normal to less than 100,000/cu. mm. was considered significant and occurred in 70 per cent of the patients. Leucopenia and thrombocytopenia were usually more severe if the patient was receiving irradiation simultaneously with F_1 or had had recent marrow depression from other chemotherapy.

The percentage of children showing each type of toxicity when given the drug intravenously is shown in FIGURE 1. Most children developed some form of toxicity within two weeks after the beginning of therapy, that is, one to three days after the completion of the course, and the toxicity usually disappeared within two to three weeks. Epistaxis or occasional local bleeding from the gastrointestinal tract have occurred when the platelet count was below 100,000/cu. mm. This usually disappeared spontaneously after discontinuation of the drug or was controlled by transfusions. Tachycardia was observed in 34 children during the course of therapy; this may have been due to anemia produced by the disease with or without bleeding, to pleural effusion, or to fever. The tachycardia may also be due to nausea and vomiting caused by the drug. Electrocardiograms were taken in 20 patients. Thirteen showed normal findings, two showed rapid rate, and five showed changes probably due to disease involvement of the mediastinum.

Aspirations of bone marrow were examined in 17 children before and after actinomycin F_1 therapy. Two showed no change, four a decrease in total cellularity, two a decrease in megakaryocytes, five a decrease in platelets, six a myeloid depression, with arrest of maturation in five, and five an eosinophilia. The eosinophilia was not related to the irradiation. The blood urea nitrogen and serum uric acid levels were studied in almost all the patients before and after therapy with actinomycin F_1. There was no elevation in the blood urea nitrogen and the serum uric acid after therapy. Urinary uric acid excretion was studied in four patients. Of these, only one patient with Hodgkin's disease showed elevation of the urinary uric acid excretion on two occasions during therapy with actinomycin F_1 associated with evidence of objective tumor regression. Patients with hemangiopericytoma, melanoma, and Schwannoma showed no objective tumor regression and no elevation of the urinary uric acid excretion.

Therapeutic effects: a total of 49 children received adequate therapy with actinomycin F_1. The objective response in children treated with actinomycin F_1 alone and with actinomycin F_1 and X-ray therapy is shown in TABLE 2. These objective responses represented a decrease in size of the tumor by actual measurements or as determined by roentgenograms and lasted for a period of at least four weeks. The first group of patients had either not received X-ray therapy or had been irradiated at least two months before receiving actinomycin F_1. Therefore, the objective response shown was considered to be due to actinomycin F_1 alone. The second group received actinomycin F_1 either simultaneously or within a period of one to two weeks before or after X-ray therapy.

Case Reports of Therapy with Actinomycin F_1 Alone

Wilms' Tumor. Of the 11 patients with Wilms' tumor treated with actinomycin F_1 alone who were suitable for evaluation, seven had definite evidence of objective response.

Case 2.: G.G., an 8$\frac{5}{12}$ year-old male, had a left nephrectomy on January 20, 1959 for Wilms' tumor with renal vein invasion. The chest X ray taken prior to operation showed a mushroom-shaped area of increased density in the left lower lung field. Six days postoperative, a repeat chest X ray showed an additional four parenchymal nodules varying between 0.5 and 1 cm. in diameter in the left lung and two nodules 2 and 3 cm. in diameter in the right apex. The patient was asymptomatic and without evidence of disease elsewhere. He was given a total tumor dose of 2100 r to the left kidney bed from January 28 to February 12, 1959, together with actinomycin F_1 to a total dose of 52 mg. (1.5 mg./kg.) in 17 days. Eight days after the beginning of actinomycin F_1 therapy, the pulmonary metastases that were not irradiated began to show improvement and by 16 days the chest X ray was clear (FIGURE 3).

From March to October, 1959, he was given daily doses of 2.0 to 4.5 mg. actinomycin KS2 orally. On October 7 an 0.5 cm. nodule was noted in the apex of the right lung. A second course of actinomycin F_1, 3.7 mg./kg. to a total dose of 90 mg., was given from October 8 to November 3 with no improvement. On December 7 a single tumor dose of 500 r was given to the right apex. The nodule disappeared but two nodules (0.5 cm. in diameter) recurred four weeks later. On January 11 a total tumor dose of 1000 r was given to the right upper lobe in four divided doses. Following X-ray therapy the two metastatic nodules disappeared and this regression lasted for two months. He is now receiving other therapy for his recurrent pulmonary metastases. Clinically he is asymptomatic 16 months after the diagnosis of Wilms' tumor with pulmonary metastases was made.

TABLE 2
NUMBER AND DIAGNOSIS OF CHILDREN SHOWING OBJECTIVE RESPONSE TO ACTINOMYCIN F_1 ALONE AND ACTINOMYCIN F_1 PLUS X-RAY THERAPY

	Total	Actinomycin KS4 (F_1)			
		Alone		+ X ray	
		Suitable for evaluation	Objective response	Suitable for evaluation	Objective response
Wilms' tumor	13	11	7	7	7
Bone sarcoma	11	10	1	5	2
Rhabdomyosarcoma	8	8	2	2	2
Neuroblastoma	6	3	1	3	3
Hodgkin's disease	2	2	1		
Sarcoma botryoides	1	1	1	1	1
Schwannoma	1	1	0	1	1
Teratoma	1	1	1	1	1
Hemangiopericytoma	1	1	1		
Miscellaneous	5	5	0	2	0
Totals	49	43	15	22	17

Comment: this patient received no X-ray therapy to his chest during the initial response to actinomycin F_1. The X-ray therapy was given only to his abdomen after nephrectomy. Therefore the disappearance of the multiple metastatic nodules in the lungs was the effect of actinomycin F_1 alone, and he had no recurrence for nine months. It cannot be certain that the oral administration of actinomycin KS2 during that time was responsible for the prolonged regression. On the other hand, the maintenance therapy of actinomycin might have been responsible for the development of resistance, because when these small metastatic nodules reappeared in the right lung while receiving actinomycin KS2 orally, a second course of actinomycin F_1 at larger total dosage did not produce tumor regression. The nodules disappeared on two occasions after X-ray therapy to that area at a relatively small tumor dose, although the effect only lasted for one and two months respectively.

Case 3: K.M., a 5-year-old girl, was noted to have an enlarged abdomen in April, 1958. The diagnosis of Wilms' tumor was made on the basis of intravenous pyelogram and the chest X ray showed pulmonary metastases. She was given irradiation to the liver to a total tumor dose of 1600 r from May 5 to May 16 and to the chest a total tumor dose of 1300 r from May 20 to June 7. The pulmonary metastases disappeared and a left nephrectomy was done on June 16. The diagnosis of Wilms' tumor was confirmed. In October she had a recurrence of tumor in the pelvic region. From November 18 to November 25 she received a total tumor dose of 1500 r to the bladder with regression of the mass. In January 1959 she had a recurrence of the pulmonary metastases. Again, she received a total tumor dose of 1500 r to the chest and 2400 r to the upper abdomen, including the liver, with tumor regression. In April, 1959, she complained of pain and weakness of the lower extremities and incontinence of urine and feces. From April 2 to April 10 she was given irradiation to the sacral spine, a total tumor dose of 1350 r for a cauda equina lesion due to metastases of the Wilms' tumor. The neurological findings gradually disappeared. In June 1959 she had a recurrence of bilateral pulmonary metastases. In addition, there was a pelvic mass 7 × 12 cm. and a left iliac node 3 × 2 cm. X-ray therapy was given to the pelvic region: a

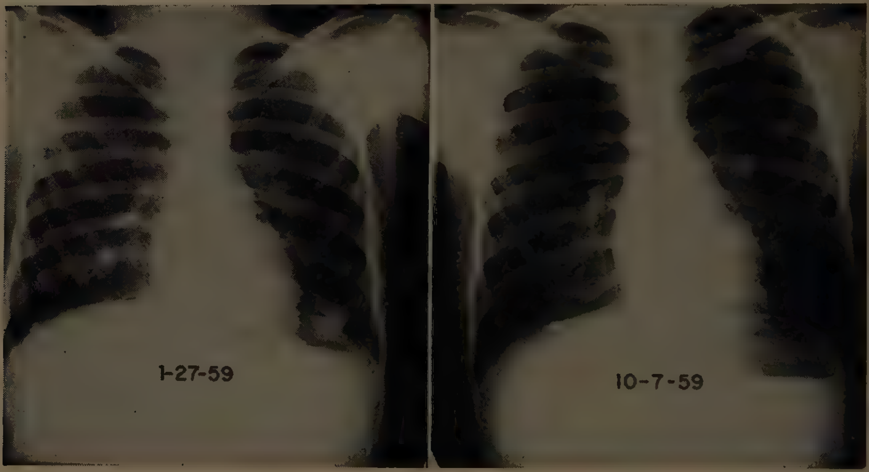

FIGURE 3. Chest X ray of G.G. (Case 2) made on January 27, 1959, before therapy (*left*). After actinomycin F_1 therapy, the bilateral pulmonary metastases disappeared; the X ray at (*right*) shows, on October 7, 1959, the first recurrence of an 0.5-cm. density on the apex of right lung.

total tumor dose of 700 r from June 5 to June 17. At the same time she received actinomycin F_1 (2.4 mg./kg.) to a total dose of 40 mg. The pulmonary metastases, which were not irradiated, improved within two weeks (FIGURE 4), and the pelvic mass disappeared completely in four weeks. She received oral actinomycin D for three weeks in July, 1959. No chemotherapy has been given since that time. Evidence of pulmonary fibrosis has been present since March, 1959, two months after the second course of X-ray therapy to the chest, and for the past four months she has had slight impairment of renal function, probably due to radiation nephritis. She has some residual weakness of the left leg. However she is active and attending school. It is now two years after the diagnosis of her disease with pulmonary metastases, and 11 months after the conclusion of actinomycin F_1 therapy with no evidence of active disease.

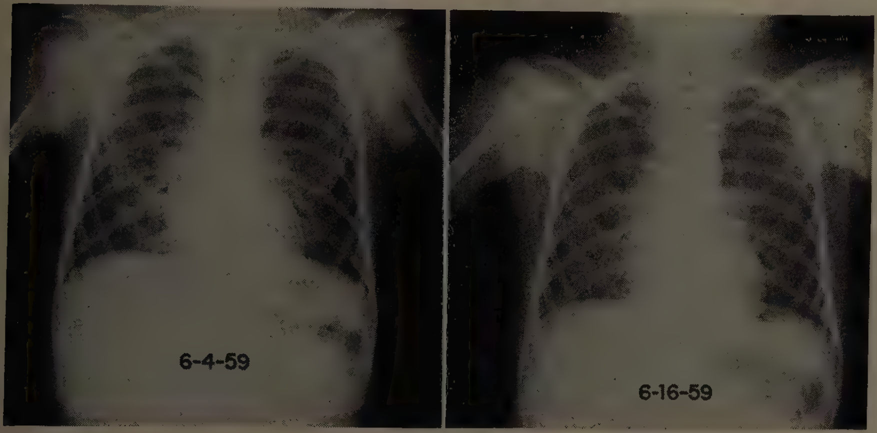

FIGURE 4. Chest X ray of K.M. (Case 3) made on June 4, before therapy (*left*) and on June 16, 1959, after therapy with actinomycin F_1 (*right*).

Comment: this child had pulmonary metastases at the onset of her disease two years ago and developed metastases to the lungs, liver, pelvis, nodes, and spinal cord. Adequate X-ray therapy was given to each of these metastatic sites with good tumor regression. In spite of the evidence of pulmonary fibrosis apparently resulting from X-ray therapy, she had recurrence of pulmonary metastases. Since no X-ray therapy has been given to the chest since January, 1959, the disappearance of the pulmonary metastases is attributed to the effect of actinomycin F_1. The chemotherapy in addition to the previous X-ray therapy might have been responsible for the long period free of recurrent disease.

Case 4: A.N. had a left nephrectomy and a diagnosis of Wilms' tumor made in August, 1958. He received X-ray therapy to his abdomen postoperative from August 20 to September 23. Eight months later he developed bilateral pulmonary metastases. He received actinomycin F_1 intravenously to a total dose of 40 mg. (2 mg./kg.) from May 4 to May 13. The pulmonary metastases disappeared two weeks after the beginning of therapy (FIGURE 5). From June 3 to July 24 he was given actinomycin D orally 1 to 4 mg. daily. However on July 17 the chest X ray showed a recurrence of the pulmonary metastases. Three courses of actinomycin D at 75 γ/kg. total dose per course given every two or three weeks with no toxicity did not produce further regression. The child expired five months after the beginning of Actinomycin F_1 therapy.

Comment: although there was remarkable regression of the pulmonary metastases after actinomycin F_1 therapy, this effect only lasted for two months in spite of the maintenance therapy with oral actinomycin D. There was no tumor regression following three courses of intravenous actinomycin D at two to three week intervals, 75 γ/kg./course, and it did not produce toxicity. The lack of response to actinomycin D may be due to the fact that actinomycin D was not given to toxicity. This patient was followed at another hospital in a foreign country.

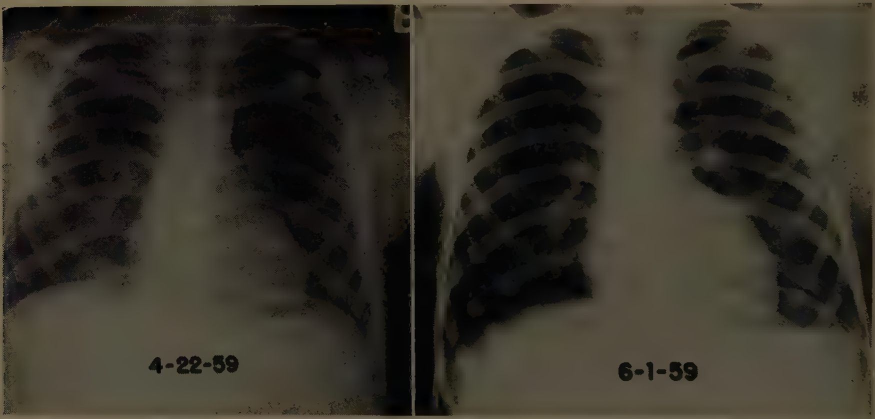

FIGURE 5. Chest X ray of A.N. (Case 4) made on April 22, 1959, before therapy with actinomycin F_1 (*left*) and on June 1, 1959, showing no recurrence (*right*).

The other four children showed regression of the pulmonary metastases for one to two months following actinomycin F_1 therapy.

Neuroblastoma. Of the three children diagnosed as having neuroblastoma suitable for evaluation and treated with Actinomycin F_1 alone, only one patient with a presumed diagnosis of neuroblastoma (but recently reinterpreted as Ewing's sarcoma) had a decrease in mediastinal and pulmonary metastases.

Case 5: G.K., a 5-year-old male, in May, 1958 had fever and pain of the right knee. In May, 1959 he began to limp, had a persistent low-grade fever, and showed marked weight loss. X ray of the right hip revealed a lytic lesion, and a mass in the right pelvis was noted. When he was first seen at Memorial Hospital in January, 1960, physical examination showed an emaciated child. The abdomen was markedly enlarged, the liver was 4 cm. below the costal margin, and a firm mass filled the right side of the pelvis, measuring approximately 4 × 6 × 6 cm. The intravenous pyelogram showed a soft tissue mass 4 × 5 cm. in the right pelvis displacing the bladder to the left. There was also a destructive bone lesion involving almost the entire right pubic bone. Chest X ray showed multiple bilateral metastases with pleural effusion on the right and a large metastatic nodule measuring 4 cm. in diameter in the left anterior superior mediastinum. Open biopsy of the pelvic mass revealed neuroblastoma. Bone marrow examination showed no extrinsic cells. From January 21 to February 8 he was given a total tumor dose of 2100 r to the right pelvis. The pelvic mass decreased in size. No X-ray therapy was given to his chest. From January 21 to April 19 he received three courses of actinomycin F_1 at two- and seven-week intervals. The dosage was 1.9, 1.7, and 1.5 mg./kg., to a total of 32.5, 30, and 27.5 mg. respectively. He developed toxicity after each course of the drug. There has been continuous regression of the bilateral pulmonary and mediastinal metastases, as shown in FIGURE 6. The pelvic mass is not palpable. The intravenous pyelogram taken on May 10 showed only slight displacement of the bladder to the left. Clinically he is asymptomatic. The pubic bone lesion has shown no change.

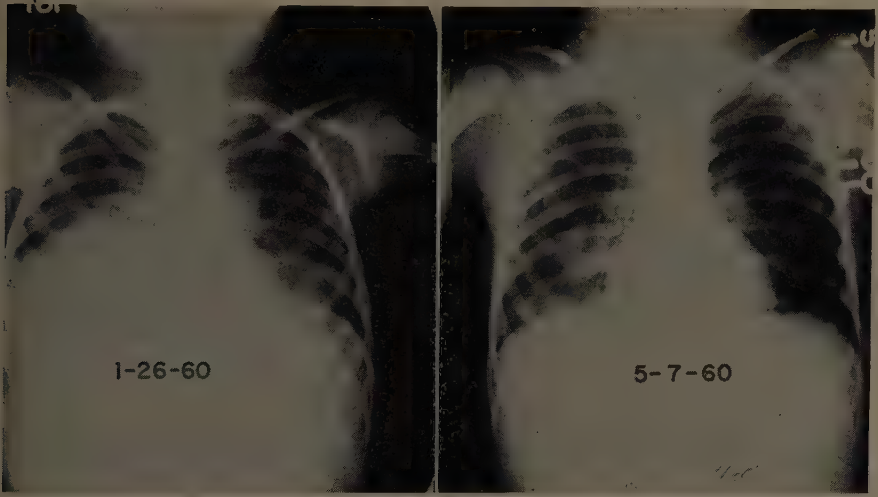

FIGURE 6. Chest X ray of G.K. (Case 5) made on January 26, 1960, before therapy (*left*) and on May 7, 1960, after 3 courses of actinomycin F_1 (*right*).

Comment: this child had bone metastases as the presenting symptom of his primary disease and had widespread metastases in his lungs and mediastinum. Although his primary site was not certain, it may be from the pelvic region. There was regression of the pelvic mass following X-ray therapy. However without X-ray therapy there was remarkable improvement in the mediastinal and pulmonary metastases. The improvement in these lesions was due to the effect of actinomycin F_1 alone.

Bone sarcoma. Of the ten patients with bone sarcoma suitable for evaluation and treated with actinomycin F_1 alone, one patient with questionable

Ewing's sarcoma had a decrease in pulmonary metastases for three months (Case 5). Two patients had definite improvement in the bone pain after therapy with actinomycin F_1.

Rhabdomyosarcoma. Of the eight patients with rhabdomyosarcoma suitable for evaluation and treated with actinomycin F_1 alone, two patients showed objective improvement.

Case 6: F.P., a 2-year-old boy, developed urinary retention in August, 1958. Biopsy of the bladder revealed rhabdomyosarcoma and he had a fixed suprapubic mass measuring 7 × 8 cm. compressing the rectum and extending up to the umbilicus. From September 16 to October 1 he was given a total tumor dose of 2500 r to the entire abdomen anteriorly and posteriorly. The mass decreased to 3 × 4 cm. in size. On October 22, twelve radon seeds were implanted into the base of the bladder. There was further regression of the tumor mass. He was asymptomatic and gained weight. In May, 1959, the mass in the perineum measuring 2.5 × 4.0 cm. recurred. Actinomycin F_1 (7.5 mg./kg.) was given from May 27 to June 27 to a total dose of 75 mg., resulting in complete disappearance of the mass. In September the perineal tumor mass measuring 3 × 3 cm. recurred. A second course of actinomycin F_1 (2.7 mg./kg.) was given from September 21 to October 2, a total dose of 27.5 mg. with tumor regression. On December 9 the perineal mass recurred again. At that time a combination of actinomycin F_1 and cobalt 60 was given to the pelvic region. The tumor mass regressed completely. It was now 20 months after the onset of symptoms; 11 months after actinomycin F_1 therapy, the patient had no evidence of disease elsewhere nor palpable mass in the pelvis. The intravenous pyelogram showed normal kidney function.

Comment: this child had extensive tumor involvement of his pelvic region at the onset of his disease. Surgical intervention was impossible. He responded first to X-ray therapy and the regression lasted for seven months. He responded to actinomycin F_1 twice, the regression lasting for two months each time. The third time he received actinomycin F_1 in combination with X ray, again with regression, and this response has thus far lasted four months.

The other child with rhabdomyosarcoma had regression of the pulmonary metastases for one month, although no change in the size of the primary tumor was noted after Actinomycin F_1 therapy.

Sarcoma botryoides. One 6-year-old male after actinomycin F_1 therapy had a decrease in pulmonary metastases for four weeks. No X-ray therapy was given to the lungs.

Hodgkin's disease. One of the two patients with Hodgkin's disease showed objective response.

Case 7: B.C., a 12-year-old male, diagnosed as having Hodgkin's disease in December, 1958, had a mass in the left lower quadrant measuring 6 × 8 cm. and a temperature of 105° F. The intravenous pyelogram showed a displacement of the left ureter opposite L_4 and L_5 by a vague 3-cm. mass and marked medial displacement in the pelvis by ill-defined 7-cm. density in the left posterior pelvis. The mass also displaced the urinary bladder to the right. From January 19 to February 16 he was given actinomycin F_1 (2.9 mg./kg.) to a total dose of 102 mg. The mass decreased to 3 × 3 cm. in size and his temperature returned to normal. This tumor regression was accompanied on two occasions by an increase in urinary uric acid levels from a normal range of about 300 mg. to over 600 mg. per 24 hours. This was not associated with elevation in the serum uric acid levels. Ten days after the completion of actinomycin therapy he was given a total tumor dose of 1000 r to his whole abdomen including the spleen and the lower pelvic mass. Complete tumor regression lasted for ten months. Later, when he developed vertebral and mediastinal nodes, he was given X-ray therapy and nitrogen mustard with good response.

Comment: this boy had disease with abdominal involvement only. There was a 50 per cent regression of the abdominal tumor mass following intravenous actinomycin F_1 alone. Because of the family situation, further courses of actinomycin were not given and, instead, conventional therapy with X ray was instituted. The tumor regression lasted for ten months and he gained 20 lb. after therapy. This effect is probably more than one would have expected from a tumor dose of 1000 r alone.

Other types of tumors. A 2-year-old girl with malignant teratoma and a 12-year-old girl with hemangeopericytoma with bilateral pulmonary metastases both showed regression of pulmonary metastases after actinomycin F_1 therapy lasting for more than one month.

Actinomycin F_1 and X-ray therapy. The additive effects of actinomycin F_1 and X-ray therapy are difficult to evaluate because of the inability to predict accurately the degree of response of the tumor in individual patients with various doses of these agents given separately. Patients were treated with approximately 50 per cent of the estimated therapeutic dose of X ray and one course of actinomycin F_1 intravenously given either simultaneously or one to two weeks apart. Wilms' tumor and neuroblastoma are considered to be radiosensitive, and actinomycin F_1 in all ten patients appeared to have enhanced the predicted degree of response to X-ray therapy. In more radioresistant tumors, although some were relatively radiosensitive, such as rhabdomyosarcoma and Ewing's sarcoma, actinomycin F_1 appeared to increase the response to X-ray therapy in two children with osteogenic sarcoma, two patients with rhabdomyosarcoma, one boy with sarcoma botryoides, one girl with malignant Schwannoma, and one girl with malignant teratoma. These results suggest that the effect of actinomycin F_1 and X-ray therapy are additive in certain responsive tumors, but we have had no convincing evidence that actinomycin F_1 sensitized radioresistant tumors to the action of X-ray therapy.

Additional observations on toxicity in adults. Anorexia, nausea, and vomiting were almost invariably seen after a course of actinomycin F_1. This effect was no indication for interrupting therapy unless, as was seen in a few cases, there was a sudden, severe, persistent exacerbation of the nausea and vomiting. When this occurred it was frequently followed in 2 to 3 days by a fall in platelet count, although the drug had been discontinued earlier. Weight loss up to ten pounds sometimes occurred after a course of actinomycin F_1, but body weight was usually promptly regained.

Bone marrow depression is the dose-limiting factor in most cases, with a platelet count fall being seen as the first and most significant change. It was observed that marrow depression was greater than expected from a given dose of actinomycin F_1 in several patients with impaired hepatic function. Great caution is required in treatment of these patients, but this correlation was not apparent in every case and the exact relationship of hepatic function and the actinomycin F_1 toxicity is not known.

In adults, the other manifestations of toxicity mentioned earlier for children, such as skin desquamation, hair loss, and oral ulcerations, are seen irregularly and are usually not severe.

A number of patients did complain of severe "light headedness." This could not be correlated with any change in blood pressure or other physical findings. It invariably disappeared within a few days after therapy was discontinued.

Therapeutic Response in Adults

One hundred and ten adult patients were treated with actinomycin F_1. Seventy-four of these patients received enough of the actinomycin F_1 to produce either sufficient toxicity to require cessation of therapy or a therapeutic response. Eighteen patients showed some objective evidence of benefit from the drug. As shown in TABLE 3, the patients with objective evidence of benefit fell into five major categories: carcinoma of the breast, large bowel, prostate, pancreas, and soft tissue sarcomas. The responses in the sarcoma group were three cases with liposarcoma and one with Kaposi's sarcoma. Forty-three patients with 16 other types of tumor showed either no benefit or only minor degrees of subjective improvement.

TABLE 3
NUMBER AND DIAGNOSIS OF ADULTS SHOWING OBJECTIVE RESPONSE TO ACTINOMYCIN F_1 (KS4)

	No. treated	No. adequate	No. notable
Breast	41	27	9
Large bowel	10	9	2
Prostate	2	1	1
Soft tissue sarcoma	10	8	4
Pancreas	4	3	2
Total	67	48	18
Other tumors	43	26	0
Total	110	74	18

Although the responses listed here were clearly worthy of note in that they showed clear-cut objective changes with or without subjective benefit, most of them were of limited significance to the patient. None of them were of more than 6 weeks' duration. The nature of these responses is illustrated in the cases cited below.

Case 8: A 66-year-old male with carcinoma of the prostate had previously been treated with orchiectomy, estrogens, and X-ray therapy to bony metastases. He had rapidly advancing disease when he started on actinomycin F_1.

He received a total dose of 46 mg., that is, 0.66 mg./kg. in 14 days. There was a decrease in bone pain and in serum acid phosphatase levels, maximally apparent one week after treatment was discontinued because of an abrupt exacerbation of nausea and vomiting. This was followed by significant thrombocytopenia. After one week of marked decrease in pain and in serum acid phosphatase levels, the patient's signs and symptoms gradually returned and he died one month later. The acid phosphatase levels were in the 5 to 9 range before therapy, fell to 1.5 units six days after treatment, and were back in the pretreatment range four days later. In our laboratory the normal range is 0 to 0.8 Bodansky units .

The two patients with carcinoma of the pancreas had somewhat similar responses. In one of these cases, marrow depression was seen after a total dose of only 20 mg. of drug or 0.30 mg./kg. The improvement in the severely deranged liver function tests lasted only a few days longer than did the thrombocytopenia.

Similarly, evanescent responses were seen in the patients with carcinoma of the large bowel and the soft-tissue sarcomas.

Of the 41 patients with breast carcinoma treated, 27 were treated adequately and nine showed objective changes worthy of note.

Case 9: a 44-year-old female (K.F.) was diagnosed in 1954 by axillary node biopsy as having mammary carcinoma. The patient refused radical mastectomy and was sequentially treated with 1 Mev roentgentherapy to the left breast and the node-bearing areas in 1954, testosterone in 1955, further X ray to both axillae in 1956, and prednisone from November, 1957, to May, 1958, at which time the actinomycin was started. While on prednisone the patient had developed an enlarging liver with the functional changes shown in FIGURE 7 and an osteolytic lesion in the right ilium. The prednisone was discontinued and actinomycin F_1 was given in two courses of 16 mg. each, that is, 0.62 mg./kg. given in 19 days.

The only toxicity was moderate nausea and vomiting. These symptoms, however, were the reason the patient gave for refusing to be readmitted to the hospital, and for refusing further treatment. She was followed in our clinic where the charted post-therapy observations showing decrease in liver size and improvement in liver function were made, but she died one month after her last clinic visit in another hospital, apparently of hepatic failure.

Case 10: a 67-year-old female (S.S.) had a right radical mastectomy in December, 1949, excision of a skin recurrence in April, 1956, followed immediately and one year later by X-ray therapy to this same area. During 1957 and 1958 she was treated with hormones. When she came to Memorial Center in January, 1958, she was receiving prednisone. This drug was gradually decreased and she was given a trial of therapy first with an androgen and then with an alkylating agent. Both therapies were without significant benefit to her skin, bone, and node involvement. When she became symptomatic with hypercalcemia, adrenal steroids did not affect the blood or urine calcium levels or the progress of her observable metastases. Actinomycin F_1, 74 mg. or 0.9 mg./kg. given in 14 days, resulted in improvement in the hypercalcemia and hypercalcuria (FIGURE 8). There was concurrent slight shrinkage of some of the skin nodules and marked subjective benefit. The course was interrupted because of severe nausea and vomiting. At no time was hematological depression observed. She received no further therapy and died two weeks later. Up to the time of her death, the calcium levels remained within normal limits.

Discussion

Actinomycins F_1 and D are toxic in man. The average therapeutic intravenous dose of actinomycin F_1 per course of treatment in children is approximately 1.5 mg./kg. in 10 days; the daily dosage is thus about 0.15 mg./kg. The dosage of actinomycin F_1 is approximately 20 times greater than that of actinomycin D. In adults, who tolerate less of both drugs on a mg./kg. basis than do children, this ratio is more nearly 10:1. The toxic effects produced by these two compounds were the same, but F_1, at the dose schedule recommended, produced more severe toxicity.

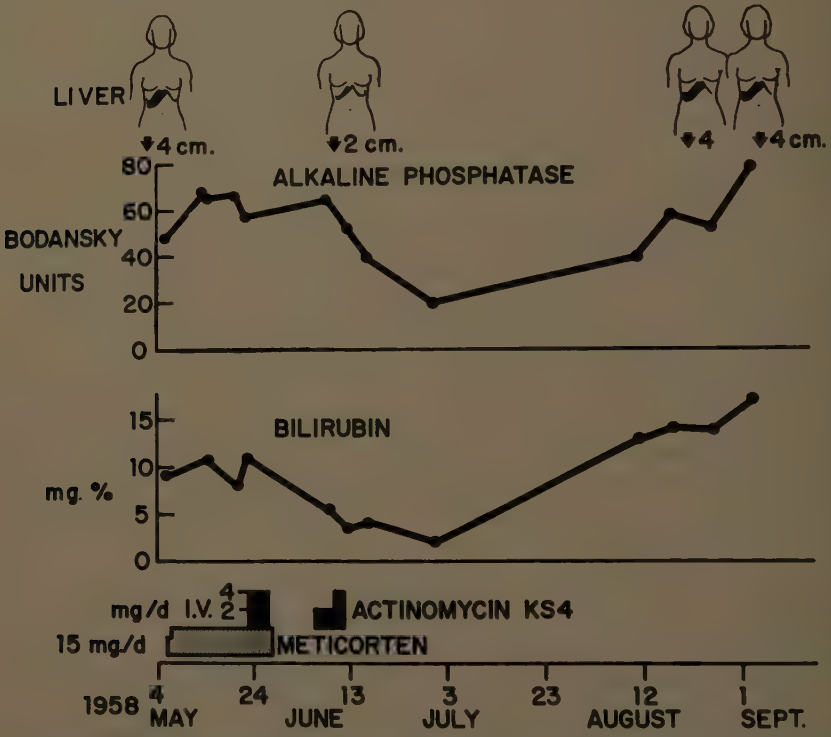

FIGURE 7. K. F., 44-year-old-female, breast cancer. The degree and duration of improvement in liver size and liver function in a patient with carcinoma of the breast, following treatment with actinomycin F_1 (KS4).

Oral administration of actinomycin KS2 (C) has not been studied adequately. In one child with acute leukemia, who was resistant to all other antimetabolite therapy, there was a transient hematological remission following oral actinomycin KS2. Further study with actinomycin KS2 in patients with acute leukemia is needed.

Comparison of the effects of actinomycin F_1 and actinomycin D. The range of effectiveness in children of these two drugs is approximately the same, in that Wilms' tumor, lymphomas, and rhabdomyosarcoma have shown better response than other tumors.

The indications for using actinomycin F_1 in certain childhood cancers is the same as that for actinomycin D. There is no definite indication for the use of these drugs, given alone, in adult patients.[5]

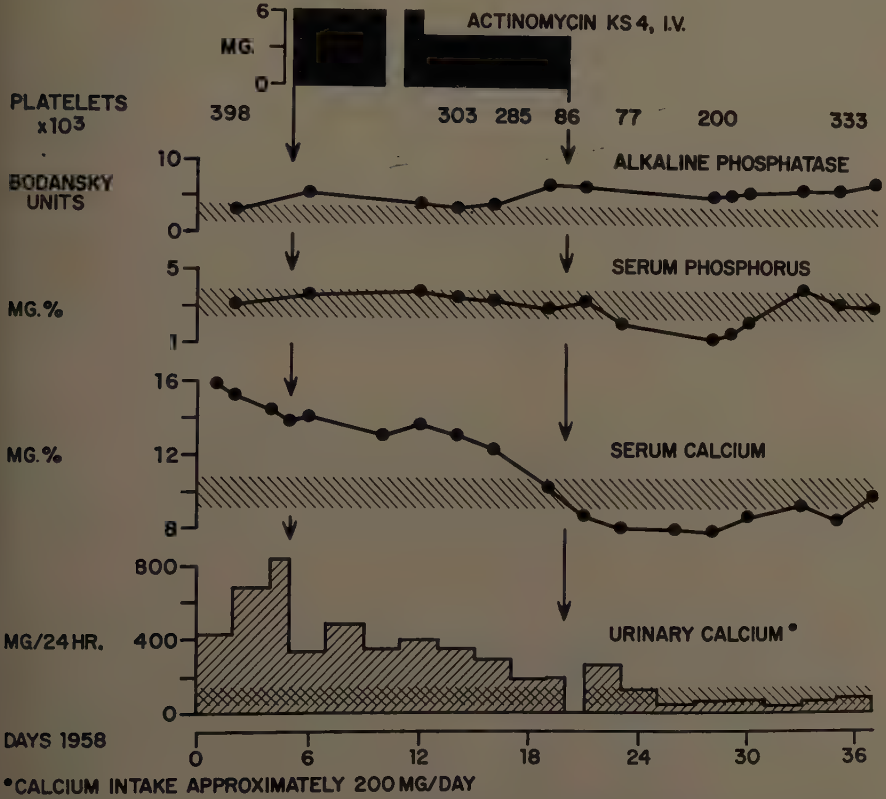

FIGURE 8. S. S. 67-year-old female, breast cancer. Chemical changes produced in a patient with osteolytic metastases from a carcinoma of the breast during treatment with actinomycin F_1 (KS4).

Summary

Three actinomycins differing in the amino acid components of their polypeptide chains have been studied: actinomycin D intravenously and orally, actinomycin KS2 orally, and actinomycin F_1 intravenously. The intravenous administration of actinomycin D and actinomycin F_1 has produced definite tumor regressions in children with cancer. Wilms' tumor and lymphomas have shown better response than other tumors. In Wilms' tumor, seven of 11 children showed tumor regression after actinomycin F_1, and seven of 20 children showed regression after actinomycin D, but these numbers are too small to permit a statistically significant comparison of the two drugs.

In Wilms' tumor the duration of tumor regression after a course of actinomycin F_1 was from one to nine months, and after intravenous actinomycin D from one to four months, except that one patient on actinomycin D has shown response in pulmonary metastases for four years.

In adults no useful effects were demonstrated in a small series treated with intravenous actinomycin D. Actinomycin F_1 had definite antitumor effects, but this drug alone cannot be recommended for the conventional management of cancer in adults. Additive effects in producing tumor regression seem to occur when actinomycin D or F_1 are combined with X-ray therapy.

The usual single course of actinomycin D intravenously in children is 75 γ/kg. of body weight divided into four or five daily doses. The average dose of actinomycin F_1 is 1.5 mg./kg. of body weight divided into 10 days, that is, about 20 times as much as the dose of actinomycin D. The toxicity of intravenous actinomycins usually develops about one to three days after the completion of the course and lasts for about two weeks.

Severe local gastrointestinal effects usually prevent adequate oral administration of actinomycin D and actinomycin KS2. The toxic effects of an orally administered actinomycin are unpredictable. Children have tolerated an oral dose of actinomycin D of 3 up to 10 mg. daily, and with actinomycin KS2 of 2 to 6 mg. daily.

Acknowledgments

We acknowledge with appreciation the supply of actinomycin D from Merck, Sharp & Dohme, Division of Merck and Company, West Point, Pa., and the supply of actinomycin KS2 and actinomycin F_1 (KS4) from G. Schmidt-Kastner and C. Hackmann of Farbenfabriken Bayer, German Federal Republic.

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CLINICAL STUDIES OF ACTINOMYCIN D

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Actinomycin D, obtained by Manaker *et al.*[1] from *Streptomyces paravallus*, has been shown to have an antineoplastic effect on several experimental animal tumors.[2] Korst and Meyer[3] reported no effect from it in three patients with Hodgkin's disease. Farber *et al.*[4] reported no effect on acute leukemia in children, but did observe a temporary effect in children with rhabdomyosarcoma, Wilms's tumor, and Hodgkin's disease. Temporary objective remissions have been noted by Wright and Safadi[5] in one patient each with Wilms' tumor, Hodgkin's disease, melanoma, lymphosarcoma, and cancer of the esophagus. No response was noted in patients with cancer of the mouth, breast, stomach, or rectum. Moore *et al.*[6] reported the effect of actinomycin D therapy on 67 adult patients with advanced cancer; temporary regressions occurred in three patients with breast cancer, three with lymphosarcoma, one with gastric cancer, one with malignant melanoma, and four with miscellaneous adenocarcinomas. These authors considered that there was little evidence that actinomycin D would be of clinical value in the treatment of advanced cancer in adults.

Clinical Studies

One hundred and thirty-two adult patients with advanced malignancies were treated with actinomycin D. The majority of the patients received each a total dosage of 75 μg./kg. body weight, administered intravenously over a 5 or 10 day period. Early in the study a few patients received 50 μg./kg. body weight over 5 days. In a few patients the 75-μg./kg. schedule was repeated when the patients' conditions permitted, but never with any dramatic results. A few patients were given weekly maintenance doses, usually 7.5 μg./kg., again with no discernible effects. The maximal amount of drug administered was 24,235 μg. over a nine-week period to a patient with carcinoma of the larynx; there was no objective response. As the therapy proceeded, the patient developed an obvious tolerance of both the toxic and therapeutic activity of the drug. The greatest amount of drug given intraperitoneally was 3000 μg. at the termination of an exploratory operation. This patient died four days postoperatively of portal vein thrombosis and a pulmonary embolus.

A summary of the results obtained in 132 patients is given in TABLE 1. There were six patients who died during or following treatment whose death could be attributed at least in part to the toxic effects of the drug. Temporary objective improvement was noted in 22 patients, or 16 per cent. The criteria of improvement included decrease or disappearance of measurable tumor masses, decrease or cessation of ascites formation, or X-ray evidence of tumor regression.

A further breakdown of the results in patients with adenocarcinomas of the gastrointestinal tract is given in TABLE 2. Temporary improvement was noted in 1 case each of carcinoma of the stomach, of the intrahepatic biliary ducts, of the gall bladder, of the ampulla of Vater, and of the rectum, and in 2 cases of carcinoma of the colon.

TABLE 3 gives the results of treatment in patients with epidermoid carcinoma. Transitory improvement was noted in one patient with carcinoma

TABLE 1
ACTINOMYCIN THERAPY FOR ADVANCED SOLID TUMORS

Disease	No. patients	Toxic death	Improved*
1. Squamous cell carcinoma, head, neck, esophagus	8	0	2
2. Adenocarcinoma, G.I. tract	55	4	7
3. Carcinoma of breast	18	1	4
4. Carcinoma of lung	8	0	0
5. Melanoma	8	0	3
6. Sarcoma	12	1	5
7. Miscellaneous	23	0	1
TOTAL	132	6	22

* Objective decrease in tumor mass.

TABLE 2
ACTINOMYCIN D THERAPY FOR ADENOCARCINOMA OF THE G.I. TRACT

Organ	No. patients	Toxic death	Improved*
1. Stomach	21	1	1
2. Liver	5	0	1
3. Gallbladder	4	0	1
4. Ampulla of Vater	1	0	1
5. Pancreas	3	0	0
6. Colon	13	2	2
7. Rectum	8	1	1
TOTAL	55	4	7

* Objective decrease in tumor mass.

TABLE 3
ACTINOMYCIN D THERAPY FOR EPIDERMOID CARCINOMA

Organ	No. patients	Toxic death	Improved*
1. Esophagus	3	0	0
2. Mouth, antrum	3	0	2
3. Larynx	2	0	0
4. Lung	8	0	0
5. Cervix	4	0	0
6. Skin	4	0	1
TOTAL	24	0	3

* Objective decrease in tumor mass.

of the mouth, one with carcinoma of the antrum, and one with carcinoma of the skin. In the patient with carcinoma of the antrum some neck metastases disappeared, while others continued to enlarge.

Actinomycin D was given to 12 patients with sarcomas; the results are given in TABLE 4. Temporary improvement was seen in one patient with leiomyosarcoma involving the liver, one with liposarcoma involving the chest wall, and three with generalized lymphosarcoma.

TABLE 5 gives the results of treatment in patients with other carcinomas, where temporary regression was seen in four of eighteen patients with carcinoma of the breast, and three of eight with malignant melanoma.

TABLE 4

ACTINOMYCIN D THERAPY FOR SARCOMA

Disease	No. patients	Toxic death	Improved*
1. Lymphosarcoma	6	1	3
2. Leiomyosarcoma	3	0	1
3. Liposarcoma	1	0	1
4. Osteogenic sarcoma	1	0	0
5. Chondrosarcoma	1	0	0
TOTAL	12	1	5

* Objective decrease in tumor mass.

TABLE 5

ACTINOMYCIN D THERAPY FOR MISCELLANEOUS MALIGNANCIES

Disease	No. patients	Toxic death	Improved*
1. Carcinoma of breast	18	1	4
2. Melanoma	8	0	3
3. Carcinoma of parotid	3	0	0
4. Carcinoma of kidney	4	0	0
5. Carcinoma of testes	2	0	0
TOTAL	35	1	7

* Objective decrease in tumor mass.

TABLE 6 gives a more detailed account of the remissions and their onset and duration for the 22 patients who received temporary benefit. Tumor regression began in some cases quite soon after completion of therapy and, in others, as late as 42 days, averaging 7 days after therapy. Remission lasted from two to 189 days, averaging 63 days. FIGURE 1 shows a liposarcoma involving the left pleural cavity and chest wall before therapy, and FIGURE 2 shows the same tumor two weeks after a 10-day course of actinomycin D; this regression persisted for three months before growth recommenced.

Actinomycin D is toxic. Its toxic effects are listed in TABLE 7. The most common side effects were nausea and vomiting, often beginning soon after the onset of therapy and persisting until its completion. The vomiting by debilitated patients may be severe enough to necessitate decreasing the daily

Table 6
Objective Response to Actinomycin D
132 Patients with Solid Tumors

Disease	No. patients improved	Type of improvement	Onset after end of therapy (days)	Duration (days)
1. Carcinoma of stomach	1	Abdom. wall tumor decreased	3	90
2. Carcinoma of liver	1	Liver size decreased	9	5 (death)
3. Carcinoma of gallbladder	1	Lymph node metastasis decreased	14	150
4. Carcinoma of ampulla of Vater	1	Abdom. wall tumor decreased	5	30
5. Carcinoma of colon	2	(1) Small-bowel obstruction relieved	21	16
		(2) Ascites formation decreased	10	60
6. Carcinoma of rectum	1	Lymph node metastases decreased	Complet.	30
7. Carcinoma of breast	4	(1) Skin tumor smaller	14	30
		(2) Skin tumor softer	5	90
		(3) Skull metastases decreased	Complet.	150
		(4) Chest wall metastases smaller	11	120
8. Melanoma	3	(1) Skull metastasis decreased	3	150
		(2) Skull metastasis decreased	Complet.	21
		(3) Skin tumors smaller	3	35
9. Carcinoma of mouth, antrum	2	(1) Neck node metastases smaller	42	42 (death)
		(2) Subcutaneous metastases disappeared	9	28
10. Carcinoma of skin	1	Neck node metastases smaller	Complet.	14
11. Leiomyosarcoma	1	Decrease in liver size	5	14
12. Liposarcoma	1	Chest wall tumor decreased	2	90
13. Lymphosarcoma	3	(1) Liver decreased in size	5	21
		(2) Neck node metastases disappeared	Complet.	189
		(3) Arm metastasis smaller	Complet.	2
Total	22	Av.	7.3	63

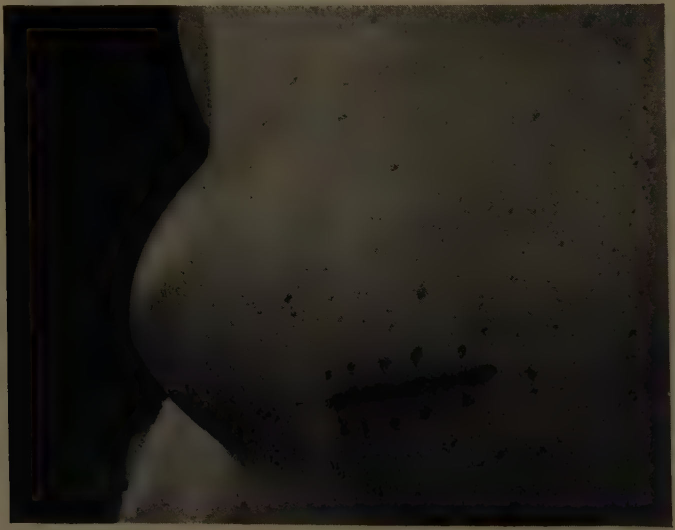

Figure 1. Liposarcoma involving left pleural cavity and chest wall, before treatment.

dose or stopping the therapy temporarily. Treatment of one patient with carcinoma of the breast had to be stopped after three days and when the total dosage was 1500 μg. Anorexia alone occurred less often. Moderately severe stomatitis was a fairly common complication; an example is shown in FIGURE 3. Alopecia was an infrequent but distressing complication, especially in women; an example is shown in FIGURE 4. This patient received

FIGURE 2. Regression of liposarcoma left chest after treatment with actinomycin D.

TABLE 7

TOXIC SIDE EFFECTS OF ACTINOMYCIN D THERAPY TO 132 PATIENTS

Disease	Anorexia	Nausea	Vomiting	Stomatitis	Diarrhea	Alopecia	Leuko-penia	Thrombo-cytopenia	Dermatitis	Epistaxis
1. Epidermoid carcinoma of head, neck, esophagus	1	2	2	2	0	2	0	1	0	0
2. Adenocarcinoma of G.I. tract	6	25	18	14	3	2	6	8	1	2
3. Carcinoma of breast	2	9	3	4	0	0	2	3	3	0
4. Carcinoma of lung	1	3	1	0	0	0	0	0	0	0
5. Melanoma	2	2	2	1	0	0	0	0	0	0
6. Sarcoma	2	4	2	4	2	1	2	1	1	0
7. Other	1	5	6	4	0	2	4	3	0	0
TOTAL	15	50	34	29	5	7	14	16	5	2

only one 10-day treatment, of 75 μg./kg. The alopecia developed promptly and persisted, although to a lesser degree, throughout the remaining months of her life, during which she received two more courses of treatment with actinomycin. Pancytopenia was infrequent; it occurred within one to seven days after completion of treatment, was often rapid in onset (24 to 48 hours), and persisted for seven to ten days. Platelet depression often appeared first

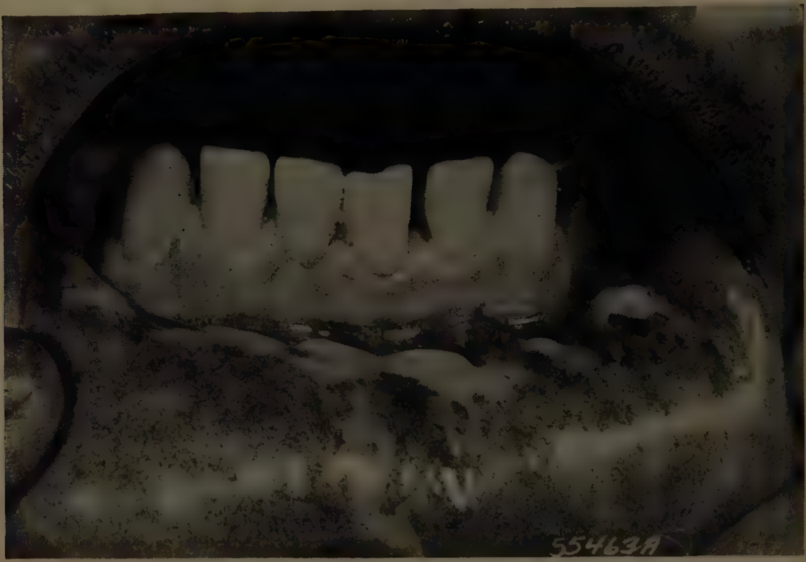

FIGURE 3. Stomatitis resulting from actinomycin D therapy.

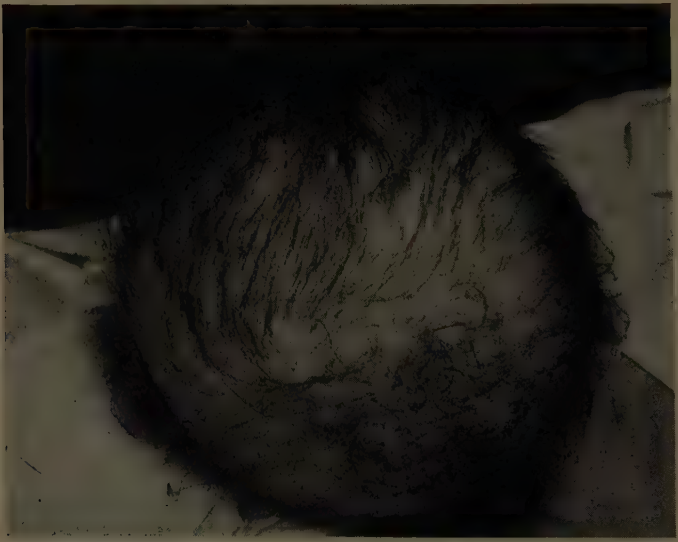

FIGURE 4. Alopecia resulting from actinomycin D therapy.

and was the first to return to normal levels. Dermatitis of an acne-pustular type, involving chiefly the face and shoulders, appeared occasionally, usually early in the treatment, and subsided spontaneously even before therapy was completed. Two patients had epistaxis without any significant depression of the blood-clotting mechanism.

Actinomycin D therapy was directly or indirectly implicated in the death of six patients. A brief résumé of these patients' clinical courses is given in TABLE 8. The patient with carcinoma of the stomach had only an omental biopsy at the time of surgery, but his postoperative course after the intraperitoneal injection of the actinomycin D at operation was stormy. Whether the surgery, drug, or tumor was responsible for the thrombosis of the portal vein remains undetermined. One patient with disseminated carcinoma of the colon, and partial small-bowel obstruction was maintained on long-tube decompression during two courses of actinomycin D therapy in an attempt to alleviate the obstruction. No antibacterial therapy was used, and with progressive debility the patient developed systemic moniliasis and hepatic failure. How much the drug contributed to this patient's debility and eventual demise is not known. A second patient with carcinoma of the colon had very severe nausea and vomiting during the five days of therapy; he became progressively weaker and died, with clinical evidence of liver failure the day following the completion of treatment. A patient with disseminated carcinoma of the rectum had a minimal toxic reaction during administration of the drug. An acute peptic ulcer developed 10 days later, and he died of hemorrhage complicated by perforation of the esophagus and by mediastinitis. The latter probably occurred during vomiting or intubation; at necropsy the esophagus showed a mucosal slough, which had contributed to the perforation. A patient with breast cancer had severe agranulocytosis seven days after completion of therapy, and died of pneumonia and sepsis, despite antibiotics and

TABLE 8

TOXIC DEATHS AMONG 132 PATIENTS RECEIVING ACTINOMYCIN D THERAPY

Disease	No. patients	No. dead	Dosage (μg.)	Therapy (days)	Days to death	Cause
1. Carcinoma of stomach	21	1	54/kg. 3000	1 (I.P.)	4	Portal v. thromb., Pul. embolus
2. Carcinoma of colon	13	2	(1) 75/kg. × 2 7220	20	18	Fat. liver, generalized moniliasis, ulcers colon, bowel
			(2) 75/kg. 4035	5	1	Liver failure, severe nausea, vomiting
3. Carcinoma of rectum	8	1	75/kg. 3150	10	20	Acute duod. ulcer, hematemesis, esophageal perf., mediastinitis
4. Carcinoma of breast	18	1	75/kg. 4297	5	7	Agranulocytosis, sepsis
5. Lymphosarcoma	6	1	70/kg. 5200	10	21	Agranulocytosis, pneumonia

supportive therapy. A patient with lymphosarcoma had severe agranulocytosis at the completion of treatment and died within 24 hours of bronchopneumonia.

Discussion

The treatment of human solid tumors with actinomycin D will result in a temporary remission in about one of six patients. Our results were best in patients with carcinoma of the breast, malignant melanoma, and lymphosarcoma. Treatment was of less value in patients with epidermoid carcinoma of the head and neck, or adenocarcinoma of the gastrointestinal tract, and it was virtually of no value to patients with carcinoma of the lung, carcinoma of the cervix, renal cell carcinoma, or testicular tumors. The variation in results—good to none—among patients having the same kind of tumor, is noteworthy.

Although some objective improvement did occur in 16 per cent of the patients, it was transitory in every case, lasting about two months. Repeated theory generally proved valueless. Tumor response seemed to have no relationship to the total dose given. Prolonged low dosages proved valueless in either inducing a remission or maintaining it.

Actinomycin D is toxic, and treatment with it can result in death. Some of our deaths may have had no relation to the drug treatment, but since actinomycin D therapy was a companion to the fact, it must share the blame. Pancytopenia or agranulocytosis, when it develops, does so rapidly, is unrelated to the total dose of the drug and may permit a fatal infection. Irreparable damage to the gastrointestinal tract mucosa may occur and contribute to a patient's death.

From the present study it is apparent that actinomycin D is of limited clinical usefulness in the treatment of advanced cancer in adults. The occasional spectacular remission is of short duration. However, the effect of actinomycin D, limited as it is, should stimulate further investigation of the clinical value of related compounds.

Summary

Actinomycin D therapy resulted in objective regression of tumor masses in 22 of 132 patients.

Best results were obtained in patients with carcinoma of the breast, malignant melanoma, and lymphosarcoma. The drug was of limited value in epidermoid carcinomas and adenocarcinomas of the gastrointestinal tract.

All remissions were temporary, beginning in approximately seven days and lasting only two months on the average.

Toxic side effects occur frequently, the more common include anorexia, nausea, vomiting, stomatitis, alopecia, and pancytopenia. These may have contributed directly or indirectly to the patients' deaths.

While actinomycin D is of very limited value in the treatment of disseminated malignant disease in adults, the results should encourage clinical studies of related compounds.

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CLINICAL EXPERIENCE WITH ACTINOMYCIN C

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Actinomycin C is a group of crystalline substances obtained from *Streptomyces chrysomallus* (*antibioticus*), a strain of *Actinomyces* found by Plotho at Göttingen. Actinomycin C was isolated by Brockmann in cooperation with Bohne and Friedrich (1954). Since 1948, C. Hackmann (1952, 1953, 1954*a*, *b*, *c*, 1955, 1956) investigated it in the research laboratories of Farbenfabriken Bayer Elberfeld for its inhibitory effect on the growth of tumors in animal experiments. Because of its special properties it was submitted for clinical testing, and the results of these trials led to its release for general use under the trade name Sanamycin. The first clinical experience with the new substance was gathered at Recklinghausen by G. Schulte, who also worked out the essentials of its dosage. Numerous clinicians have worked with the drug since. Of the investigators outside Germany, I may mention the French workers, Ravina and Pestel (1954, 1955), who investigated chiefly the range of indications but also reported new guiding principles for the dosage of the preparation. We have worked with the drug again and again for almost a decade and have tried to arrive at a sound definition of its clinical value.

The administration of actinomycin C was at first possible only by the intravenous route, which has remained the most used and most effective. It is important for intravenous administration that the substance be dissolved only immediately before use and that the injection be given slowly. Actinomycin C should never be mixed in the syringe with other drugs—for example, vitamins—which may affect its efficacy. Its dosage has undergone frequent variations in the course of the years. The average dose for intravenous administration is 200 μg. daily. This may be temporarily doubled. The total dose for a whole course of treatment is generally 10,000 to 15,000 μg. The first clinical effect is usually seen after 3000 to 5000 μg., but lower doses may suffice in some patients, while higher doses may be required for others. Administration by continuous drip infusion considerably enhances the clinical effect in many cases. Ravina and Pestel (1955) were the first to report this mode of intravenous administration, for which they gradually raised the daily doses to 600 or even 800 μg. dissolved in 250 to 500 ml. normal saline. My associates and I have used the continuous intravenous drip in many cases—often with very good results—but we seldom gave more than 600 μg. daily. When it had been found that actinomycin C is also absorbed and better tolerated on rectal application, its use became possible on a yet larger scale. Rectally, much higher doses, 500 to 1000 μg. daily, are given and can be continued for prolonged periods, unless side effects occur. However, it is unlikely that intravenous administration can ever be dispensed with in the very severe cases. The dose of actinomycin C remains unchanged when the drug is used in combination with other drugs (cortisone, nitrogen mustard, or triethylenemelamine) or with X rays.

Actinomycin C is generally well tolerated; its tolerance at least compares well with that of other cytostatic substances. Intravenous administration may occasion irritation of the vein and thrombosis, and it is therefore advisable to give it in high dilution. Mild disturbances of general well-being, with impairment of appetite, retching, and a tendency to diarrhea are frequently seen and may become severe enough to necessitate discontinuation of the treatment. The typical side effects observed in a course of treatment with actinomycin C are mucosal changes. They occur comparatively frequently in the form of burning of the tongue, gingivitis, and stomatitis; less frequently as laryngitis, tracheitis, gastritis, enteritis, or vaginitis. Frequency and intensity of side effects depend on both the dose given and the patient's general state. The effects occur rarely with daily doses of 200 μg., more frequently with 300 μg. daily, and almost regularly with 400 μg. daily. Continuous drip infusion, as recommended by Ravina and Pestel (1954, 1955), does not safeguard against side effects, but rectal application in the form of suppositories reduces them to a minimum. We gave a woman with reticulosis 160,000 μg. Sanamycin as suppositories in a course of treatment lasting four months; no side effects occurred. Lymph node swellings considerably regressed during this treatment and then were maintained at tolerable size. To reduce side effects it is advisable to supplement actinomycin C treatment with high doses of vitamins B, C, and A, as pointed out also by Ravina (1953). Other side effects that have been reported to occur occasionally are transient loss of hair (Ravina and Pestel, 1954*a*, *b*, and *c*; Bertrand-Fontaine *et al.*, 1954; Croizat, 1954) and, very rarely, liver damage (Nitsch and Thein, 1955; Huguenin *et al.*, 1954). We observed a case of polyneuritis, which was probably of allergic origin and pyrexial reactions due to hypersensitivity. The major advantage of actinomycin C over other cytostatic treatments is that it almost never causes depression of the bone marrow in cases of leukopenia or thrombocytopenia, nor affects spermatogenesis.

The results of animal experiments made by Hackmann, who observed chiefly a reduction in the size of lymphatic tissue under the effect of actinomycin C, caused him to suggest that it be employed especially for diseases of this system. Most of the reports available, therefore, deal with the treatment of Hodgkin's disease. A survey of the essential points of experience gained in the treatment of this disease, as they have become known to us and have been reported in the literature, is presented in TABLE 1. Although reports are numerous, they do not easily allow a satisfying interpretation of the value of the drug in the disease, since most workers gave simultaneously other drugs or X rays and did not clearly separate the cases they had treated with actinomycin alone from those in which the drug was used in combination. The most comprehensive review for this indication, that of Schulte's (as completed by Vogelsang and Többen, 1956), also is not very conclusive in this respect, because these authors treated almost all their patients with actinomycin C plus X rays. A further difficulty in evaluation resides in the fact that the effect of actinomycin C—like that of other drugs or X rays—is dependent on the stage of the disease at which it is used. In accordance with the practice of many other workers, we first gave it only for advanced cases, in which the response is naturally inferior to that of new. Later we extended its use to newly diseased patients and then obtained better results. During the past eight years we have treated

TABLE 1

TREATMENT OF HODGKIN'S DISEASE WITH SANAMYCIN

Authors	Treatment (no. of cases)			Dose (µg.)		Results			Side effects	Remarks
	Total	Sana-mycin alone	Combined	Daily	Total	Good	Doubt-ful	None		
Schulte, 1953	100	43	X ray, 57	300	10,000	39	57	4		Recent cases responded best
Ritter, 1954	1	1		500	15,000	1			Tongue burning	
Martin, 1954	24		X ray, 24	400	10,000	24			Gingivitis	
Kipping, 1954	3	2	X ray, 1	500	20,000	1	2			
Johne and Kroher, 1954	1	1		200	4,950	1				
Bertrand - Fontaine *et al.*, 1954	15	15		400	12,000	1	2	12	Mucosa inflamm., hair loss	"No significant effect"
Huguenin *et al.*, 1954	21	19	Cortisone, 2	400	10,000		4	17		Even recent cases not always responsive
Croizat, 1954	16								Icterus, hair loss	"Very doubtful effect"
Janbon, 1954	5	5		400	7,000		5			"Disappointed"
Gernez-Rieux and Goudemand, 1954	3			400	10,000	1		2	Mucosa inflamm.	"Unsatisfactory"
Olmer, 1954	7		X ray or cortisone in some cases	200	7,800		3	4		
Martin and Munier, 1954	12	12		250	9,300	1	5	6	Diarrhea	
Börnstein and Stein, 1954	1	1		200	9,000	1				
Storti and Pederzini, 1954	6		X ray or cortisone	200	5,000		6			
Den Boon, 1954	1	1		200						
Schmidt *et al.*, 1955	8			400	17,500	2	3	3	Mucosa inflamm., vomiting	
Bollag and Essellier, 1954	8							8		"No effect"
Ravina and Pestel, 1955	9	6	X ray or cortisone in some cases	800	10,000	4	2	3	Mucosa inflamm., hair loss	In some cases, continuous drip
Osten and Zademack, 1955	6	6		300	40,150	3	3			
Nitsch and Thein, 1955	1	1			7,800		1		Liver damage	
Magnus and Zeitler, 1955	13		X ray	200	8,000	13				
Field *et al.*, 1955	3						3			
Vogelsang and Toebben, 1956	128		Mostly with X ray							No details
Haensch, 1956	2	2		200	6,000					

a total of 54 cases of Hodgkin's disease with Sanamycin. In 16 of them the disease was so far advanced that it no longer responded to any treatment, whether by drugs or irradiation. Of the other 38 cases, 20 were treated with Sanamycin alone, 10 with the drug in combination with X rays, and 8 in combination with nitrogen mustard or a similar drug or with prednisone. The results are shown in FIGURES 1 and 2. The rating "good" is for cases in which all symptoms regressed and a complete remission was achieved. In the cases with "questionable" success some of the symptoms improved and the

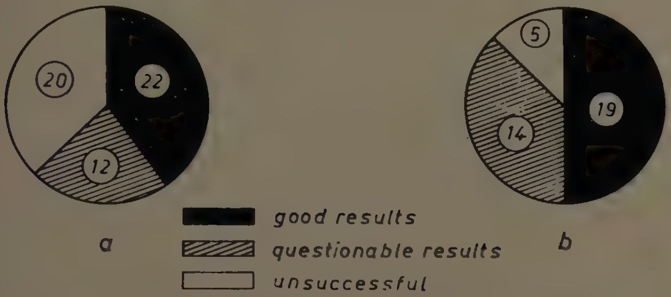

FIGURE 1. (*a*) Total results of Sanamycin treatment of Hodgkin's disease (54 cases). (*b*) Results in cases not refractory (38 cases).

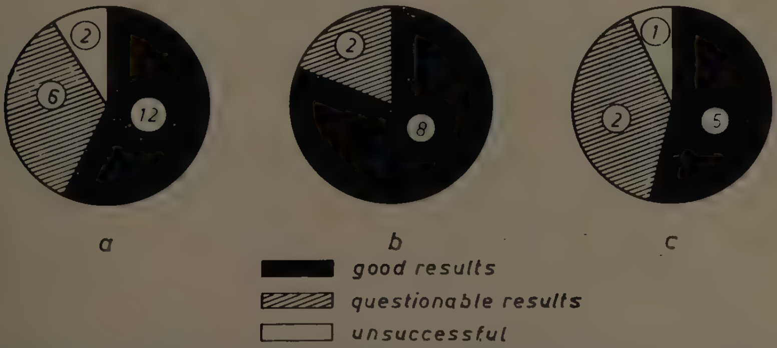

FIGURE 2. Treatment in cases of Hodgkin's disease not refractory to therapy. (*a*) Sanamycin alone (20 cases). (*b*) Sanamycin and X rays (10 cases). (*c*) Sanamycin and other drugs (8 cases).

affected lymph nodes were reduced in size, while other signs—for example, fever, general state, blood condition, and erythrocyte sedimentation rate—were not influenced. The last group of patients was not benefited at all. No reference is made to the duration of remissions. It is hardly possible to cite valid data for purposes of comparison with other therapeutic measures. We have gained the impression that remissions are, on the whole, of shorter duration after exclusive actinomycin C treatment than after irradiation or nitrogen mustard. In a few cases, subsequent to clinical treatment, we continuously gave Sanamycin suppositories, but this did not appear to succeed in preventing relapses. It seemed the patients became "accustomed" to the preparation in the course of time, with ensuing reactivations of the disease.

A comparison of our results with those reported in the literature shows fair agreement in some respects. Some authors were no doubt sceptical of the value of actinomycin C (Bollag and Esselier, 1954; Bertrand-Fontaine *et al.*, 1954; Huguenin *et al.*, 1954; Croizat, 1954; Janbon, 1954; Gernez-Rieux and Goudemand, 1954; Olmer, 1954; Martin and Munier, 1954; and others), but all who worked intensively with it and did not let themselves be discouraged by initial failures obtained results similar to ours. Actinomycin C has, according to our experience, its rightful place in the treatment of Hodgkin's disease, in particular the primarily leukopenic cases, for which one hesitates to give other cytostatic agents. Milder cases and those of recent origin respond better than pretreated and severe cases, but even the latter will respond successfully to a course of Sanamycin treatment.

TABLE 2

LYMPHATIC LEUKEMIA TREATED WITH SANAMYCIN

Patient	Before treatment			After treatment			Reduction of lymph node and spleen swelling	Sanamycin dose (μg.)
	Hb	Eryth.	Leukoc.	Hb	Eryth.	Leukoc.		
G.O.	12.0	4.2	78,864	12.5	3.7	97,650	+	4,600
A.K.	4.8	1.26	9,360	4.6	1.3	11,040	0	400
G.G.	16.5	5.9	31,960	15.4	4.1	51,300	++	5,950
L.K.	10.0	3.1	97,084	11.8	3.8	98,800	+/0	1,700
A.K.	12.0	3.0	4,278	14.0	4.2	2,496	++	11,200
H.M.	9.1	2.8	147,000	10.7	2.5	153,900	++	9,000
J.H.	6.8	2.4	3,120	10.6	3.5	1,950	++	5,750
G.P.	9.5	3.7	2,540	12.8	4.1	550	+	7,000
G.G.	15.0	4.5	82,450	14.4	3.7	76,440	+	8,600
H.St.	12.3	4.2	32,000	12.5	4.3	35,000	+	20,000*
K.H.	15.8	4.8	23,800	15.2	4.6	25,600	+	25,000*
A.K.	13.9	3.9	53,700	13.7	4.3	52,000	+	6,200
L.P.	12.7	3.5	14,000	12.1	3.5	17,300	+/0	5,900
A.Z.	14.5	4.3	5,700	15.2	4.5	8,000	++	32,000*

* Rectally.

Another, in our opinion, equally important indication for actinomycin C is in the treatment of chronic lymphatic leukemias, especially the aleukemic forms with peripheral leukocyte counts below 30,000/cu. mm. and multiple swelling of lymph nodes, to which X rays cannot be given because, for a satisfactory effect to be achieved, too high a radiation dose would be needed. Table 2 shows the results we have obtained from 14 cases of lymphadenosis. Lymph node swellings or tumor of the spleen were almost regularly reduced. The treatment had no notable effect on the leukocyte counts in most cases. The effect of Sanamycin therefore is similar to the effects of cortisone, prednisone, and related drugs, but it does not cause the same side effects (adrenal atrophy, cushingoids, retention of water, or steroid diabetes). Lymphadenosis is therefore an indication for actinomycin C by itself, and it is surprising that few reports of its use have appeared until now. Schulte (1952), Janbon (1954), and Magnus and Zeitler (1955), who have treated several cases, also obtained good results.

Giant follicular lymphoblastoma (Brill-Symmer disease) is another condition that actinomycin C affects with a regression of lymph node swellings and spleen enlargement, but the results depend on the stage of the disease at which it is given. Good results were obtained in some cases by Ravina and Pestel (1954, 1955) and by Riegel and Krueckenmeyer (1957), while no success was seen by Olmer (1954) and a failure was experienced by Riegel and Krueckenmeyer (1957) with the disease in a late stage. We observed regression of lymph node swellings in two cases, and an improvement in general condition.

Varied results also have been reported for reticuloses, reticulosarcomas, and lymphosarcomas. We observed transient improvement in one case of reticulosis, while two others and two of reticulosarcoma were not influenced. The case reported by Ravina and Pestel cannot be conclusively evaluated, since treatment was combined with X-ray irradiation. Croizat (1954) saw no clear-cut effect, and neither did Croizat (1954), Huguenin *et al.* (1954), Olmer (1954), or Ravina and Pestel (1954, 1955) in lymphosarcomas.

Other blood diseases, such as chronic myelogenic leukemia (Magnus and Zeitler, 1955), acute leukemias (Gernez-Rieux and Goudemand, 1954; Janbon, 1954) and plasmocytoma (Schultze, 1958), did not respond generally to treatment with actinomycin C. Albrich and Hefeli (1956), however, achieved a surprising success in a case of plasmocytoma: after 6000 μg. Sanamycin had been given, ostealgia disappeared, protein values and anemia were improved, and the erythrocyte sedimentation rate had declined from 116 to 16 mm. in one hour. Schultze (1958) noted chiefly subjective improvement in a patient with plasmocytoma after 10,000 μg. Sanamycin. We observed no notable subjective or objective improvement in three plasmocytoma cases.

The results of Hackmann's studies of malignant tumors in animals suggested the use of actinomycin C in the clinical treatment of such conditions. Trials have been successful only in exceptional cases (Croizat, 1954; Gernez-Rieux and Goudemand, 1954; Huguenin *et al.*, 1954; Janbon, 1954; Olmer, 1954; Schmidt *et al.*, 1955). The partial successes reported by Ravina and Pestel cannot be reliably interpreted, because Sanamycin was given subsequent to surgical removal of the tumor or with other drugs or X rays. By direct instillations into the peritoneum and the urinary bladder, however, these investigators achieved clinical and local improvement in an ovarian carcinoma and peritoneal metastases and in a diffusely growing carcinoma of the bladder. Schmidt and Watrin (1955) obtained surprising success in a case of hypernephroma with pulmonary metastases: after surgical removal of the primary tumor, the extensive metastases in the lung completely disappeared during Sanamycin treatment for 18 months. We observed no cytostatic effect of actinomycin C in four patients with carcinoma (of the choroid membrane, of the prostate with metastases, of the stomach, and of the gallbladder).

The marked effect actinomycin C has on lymphatic tissue naturally gave rise to the question of whether the drug would influence antibody formation and be of value in the treatment of diseases due to allergy or pathological antibody formation. Businco (1955), Ravina and Pestel (1954, 1955), and also Meythaler and Fischer (1958) gave Sanamycin to patients with bronchial asthma and with asthmoid emphysematous bronchitis. They reported a favorable effect in about one half of the patients. We have not yet used Sanamycin for diseases of this kind, but we gave it to three patients with acquired hemolytic anemia due to incomplete heat agglutinins. Although we did not, on the whole, observe a notable effect, in one case a Coombs' test result became temporarily negative. Further clinical and experimental studies in this direction appear justified. If it were proved that actinomycin C actually inhibits antibody formation, further important possibilities would be opened up for its use.

Summary

The clinical experience with Sanamycin (actinomycin C), as reported in the literature and as gained by my associates and myself, is reviewed. Owing to its almost selective cytostatic effect on lymphatic tissue, actinomycin C is indicated especially in diseases of this system, in particular, Hodgkin's disease and chronic lymphatic leukemia. Since it does not cause depression of the bone marrow, leukopenic diseases are its special field of indication. The effect of actinomycin C is less intense than that of similar cytostatic agents, especially nitrogen mustard. Remissions are usually of shorter duration after Sanamycin treatment than after nitrogen mustard or X-ray treatment, but Sanamycin offers the advantage of being less toxic. The absence of inhibitory effect on the bone marrow and of spermatogenesis is pointed out. The side effects of Sanamycin treatment are discussed in detail. Sanamycin is found to be of little effect in cases of malignant tumors. Its cytostatic effect on the lymphatic system suggested the possibility of an inhibition of antibody formation, and thus the question whether it may be beneficial in diseases of allergic or immunological origin. Some encouraging experience has already been gained in this direction. Further experimental studies, in particular with regard to the question of effect on antibody formation and the modification of actinomycin C, may succeed in widening the field of its clinical use and rendering it more effective.

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THE CLINICAL APPLICATIONS AND METHODS OF ADMINISTRATION OF ACTINOMYCIN IN THE TREATMENT OF DIFFERENT TUMORS

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For many years now it has been established that the extracts from certain species of *Actinomyces* have, in laboratory animals as well as in humans, the property of inhibiting or destroying certain elements of the lymphatic system and tumoral cells. Many varieties of the extract actinomycin are now known, and their list is probably not concluded. The first one discovered, derived from *Streptomyces antibioticus* 3435 and prepared by Waksman and Woodruff[1] in 1940, was called actinomycin A. Of the many other varieties subsequently discovered, only two (actinomycins C and D) have been of current use in therapeutics. We have had opportunities of using both: actinomycin C in the form of Sanamycin Bayer, and actinomycin D, kindly supplied to us by S. Waksman.*

While these two varieties have entirely different chemical, physical, and natural properties, their therapeutic and toxic actions are almost identical; in many cases we have substituted one for the other without observing significant changes in either clinical or side effects.

Of the 200 patients we treated, two thirds received actinomycin C; one third, actinomycin D.

In this report we shall abstain from making references to chemical or biological problems and shall confine ourselves to the therapeutic action of both substances.

Because of its extreme vulnerability, we thought it preferable to administer the drug in perfusions of 200 to 600 μg. in 200 ml. of saline solutions. The perfusions, which last an hour and a half, require careful checking of the blood *p*H, which must remain absolutely neutral, as well as complete protection of the bottle from light. In addition, and still with a view to protecting the patients from the toxic properties of the drug, we gave, at the end of each perfusion, an injection of vitamins B_1, B_6, pellagra preventive, and pantothenate of calcium. Although this technique is perfectly logical and in favor with many authors, it did not seem to give better results than the rapid injection of 200 to 400 μg. of actinomycin in 4 or 5 ml. of distilled water or saline solution.

We endeavored to establish dosages proportionate to the patients' weights. Doses were found to be identical for actinomycin C and D; we prefer doses not exceeding 3 to 6 μg./kg., that is, 0.125 mg./kg. for one treatment. These figures are certainly much lower than those given by Farber,[2] who fixed at 8 or 9 μg./kg./day the amount to be given to children, with a maximum of 675 μg./kg. in a treatment of 125 days. Burchenal and C. Tan (personal communication) give 15 μg./kg. for five days only. We had opportunities to observe only a few children's cases, but in such instances we gave doses identical with those given to adults. We noted, however, that children, as a rule, showed a better tolerance of the treatment.

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Our experience seems to indicate that 8000 μg. is the maximal dose that may be administered intravenously; when this is exceeded, the patient frequently presents intolerance phenomena that interfere with the treatment.

In some cases treatment had to be resumed after 10 or 12 days, but the average interval between two series of treatments was two or three weeks, with the possibility of having to prolong this interval several months. Unfortunately, a certain number of patients will finally develop a resistance that, added to a progressive obliteration of the veins, hinders the cure. The oral or local administration of actinomycin will make it possible to continue the treatment over a longer period.

The Intravenous Administration of Actinomycin

We are reporting in this paper certain results obtained from 200 patients observed since the beginning of 1954.

Particular attention was given to the treatment of malignant tumors. Only few hemopathic conditions were observed. In some instances the progress of the disease was not affected by the treatment.

Three cases of myeloid leukemia showed considerable improvement, which persisted several weeks to several months, with a decrease of two thirds of the leukocyte count.

We obtained an unexpected recovery of a patient with giant follicular lymphoma (an exclusively splenic form of Brill Symmer's disease). The first symptoms had appeared in 1949. In 1951 a splenectomy had resulted in temporary improvement. As early as 1952 the patient's condition became alarming again and, at the beginning of 1954, progressive anemia set in and resisted all therapeutic measures; simultaneously, hematomata and intestinal hemorrhages developed, which were impossible to control. There was deterioration of the blood, which showed anisocytosis, poikilocytosis, anisochromia, and an increased number of leukocytes. At this stage we thought we were going to lose the patient.

In March 1954 we gave him 7200 μg. of actinomycin for a fortnight. The patient improved considerably until December when, again, accidents occurred. Treatment was resumed and the patient received 6200 μg. of actinomycin, which brought about a new improvement. The same treatment was given again in 1957 with the same results. The patient continued gradually to improve; he gained some weight, was able to work, and now may be considered actually cured. His recovery obviously is due to actinomycin; all other treatments previously administered had failed.

Of ten cases of Hodgkin's disease, two treated in 1955 and 1957 have had no relapse. One of the two had received several series of treatment with 7000 μg.; the other, only 2000.

A young woman bedridden with Hodgkin's disease since 1951 and unimproved by radiotherapy, in 1953 was completely transformed by 1000 μg. of actinomycin. Later she received several preventive doses of the drug and was able to lead a normal life for several years. There were flare-ups of the disease in 1956 and 1958, and each time these were checked with 7000 μg. Unfortunately, obstruction of the veins finally prevented us from continuing the treatment and the patient died in 1959. In this case also, actinomycin had prolonged the patient's life by several years.

Two other patients with Hodgkin's disease survived three and four years, although at the time their treatment was begun they were in advanced conditions. Two patients showed only slight improvement, and three others showed no improvement.

We gave actinomycin to 16 patients who had been operated on for brain tumor. All had presented serious cases, due either to resection that was not extensive enough or to the histological nature of the tumors, which had made us fear early recurrences. In most instances actinomycin showed poor results; in some, no results. Nevertheless five patients showed temporary remission with disappearance of all or almost all clinical symptoms for several months.

Treatment of sarcomas and epitheliomas. In spite of numerous failures in the treatment of sarcomas and epitheliomas we obtained several results of some value.

For example, one patient presenting esophageal carcinoma with almost complete dysphagia received some food after administration of a 6400-μg. dose, and was able to start working again. Six weeks later, however, he relapsed. He was improved again by a 2300-μg. dose. He was to die after six months, but actinomycin twice permitted him a valuable retrocession that lasted for some time.

Certainly, the most spectacular results were obtained in multiple pulmonary metastases of carcinomas of various histological origins. The patients presented not only clinical but, in some cases, radiological retrocession.

This was observed in a man of 22 years with multiple pulmonary metastases that almost disappeared and then remained as they were for 18 months. He had been given 31,000 μg. of actinomycin.

A woman of 65 years, presenting bilateral pulmonary cancerous dissemination of granular form associated with hemorrhagic pleuritis and accidents consequent to a latent pancreatic carcinoma, was greatly improved by a dose of 3600 μg. administered over three weeks. We obtained the disappearance of the pleuritis and the pulmonary conditions. The patient died eight months later. Post-mortem examination showed the presence of several pleural neoplastic nodules but no pulmonary lesions.

Several patients with kidney and uterus carcinomas and pleural and mediastinal metastases were improved only temporarily, although appreciably, and showed retrocession of the ganglionic masses in the remission of pains, disappearance of pleural effusion, and improvement in weight and general condition.

Several cases of sarcoma were also improved:

In a child of 11 years presenting a rapid evolution of an enormous thoracic angiosarcoma, actinomycin given in a dose of 4200 μg. afforded the symptoms of survival for 11 months under circumstances of normal life.

A patient with a subhepatic lymphosarcoma that was impossible to operate on resisted the perfusion of nitrogen mustard. His general condition was particularly bad, with emaciation, asthenia, icterus, and digestive symptoms. A treatment of actinomycin D, given by perfusion in doses of 200 μg./day, gave a spectacular improvement after only 1000 μg. The first treatment with 6000 μg. was followed four months later by preventive treatment with 2000 μg. After two months the patient, considering the recovery complete, went on vacation, apparently still in perfect health, but died from acute pulmonary phenomena several days after taking a bath in cold sea water.

A 9-year-old girl had a sarcoma of the third rib with pleural metastases. The tumor was removed March 1955. Following the operation she was given 1900 μg. of actinomycin. No deep X-ray treatment was applied. The patient had a slight relapse in 1959 and was treated surgically. She is all right to this day.

Local Treatment

Unquestionably favorable results may be obtained by placing actinomycin C or D in direct contact with certain tumoral lesions. This may be due to the fact that this method permits a greater deposit of cytostatic elements at the precise site of the lesion, than do intravenous injections.

In carcinomata of the pleura, the peritoneum, and the pericardium, we inject the actinomycin directly into the serosa. In cancers of the bladder we inject the medication into the bladder and leave it in contact with the lesion as long as possible. As a rule, we inject a dose of 200 μg. of actinomycin dissolved in 20 ml. of saline solution, after evacuation of one half or three quarters of the fluid. We find that the total dose needed for local treatment (usually 2000 to 3000 μg.) is always smaller than in other methods of administration. Nevertheless we frequently associate the local treatment with the intravenous administration of 6000 to 8000 μg.

While local treatment may in some cases have little or no action, in others it may produce considerable and more or less enduring improvement. Our best results were those obtained in the treatment of ascites of cancerous origin.

To illustrate our theory, we report the case of a woman who presented an ovarian carcinoma extending to the pelvis, the epiploon, and the peritoneum. She was admitted to the hospital December 27, 1953, in very bad condition and unable to leave her bed. Voluminous cancerous formations filled the pelvis and the abdomen. Ten times between December 30, 1953 and February 18, 1954 the removal of two liters of hemorrhagic ascites fluid was followed by the local administration of 200 μg. of actinomycin. This brought about a total change in the patient's condition: at the end of seven weeks all malignant growth and ascites had disappeared. Following this, the patient received a series of intravenous injections of actinomycin, to a total of 6000 μg. She remained symptom-free for nine months, when again she presented some ascites and a circumscribed mass, which was removed surgically. After a further administration of 10, 400 μg. of actinomycin the ascites again disappeared, and the patient stayed in good health for three years, being able to resume an almost normal life. On several occasions recurrences of ascites were cured by further administration of actinomycin. The patient died in 1958 from cardiorenal complications, but the post-mortem examination showed new peritoneal metastases.

In 1955 we admitted a 49-year-old woman presenting an ovarian carcinoma with general peritoneal carcinosis. An exploratory laparotomy and a biopsy corroborated this diagnosis.

From January 10 to 24, 1955, the patient received 7000 μg. actinomycin by intravenous injections, which resulted in reducing the volume of ascites and improving her general condition, although bringing slight temporary stomatitis and alopecia.

After this first treatment and for 12 months the patient, owing to vascular sclerosis that obliged us to stop intravenous injections, received 200 μg. actinomycin administered into the ascites liquid after punctures. The evolution was slow, and for a long time the patient was able to lead a normal life, even undertaking long trips, but after some months the effects of the therapy obviously decreased and, although local conditions were still satisfactory with only a slight ascites and abdominal masses neatly reduced, the state of the patient slowly worsened and she died after a year of treatment.

A woman of 70 years with a primary carcinoma of the gallbladder was apparently cured for a year by a cholecystectomy when, in the first days of July 1959 she presented a progressive icterus, soon followed by a generalized pruritus and then by the beginning of ascites with peritoneal and subcutaneous nodules. Her general health was greatly affected. Ten intraperitoneal injections of 200 μg. actinomycin given each week suppressed the ascites and improved her general condition without, however, having any effect on the icterus. In November the ascites appeared again and new injections in the same amounts gave new improvement but failed to suppress completely the ascites. Unhappily, the patient's veins being in poor condition, it was impossible to combine intravenous injections with the previous treatment. However, the patient seemed to be definitely improved for some months with local treatment alone.

A woman of 65 years presented a carcinoma of the cervix and underwent a hysterectomy in 1957. In March 1959 new troubles appeared in the form of hemorrhagic ascites and a bilateral inguinal adenopathy. A treatment of 16 intraperitoneal injections administered at the rate of two a week and 200 μg. given orally every other day produced an improvement that lasted several months, with retrocession of the adenopathy and disappearance of the ascites.

In 10 patients actinomycin administered by intrapleural injections gave no satisfactory results. The treatment often produced thoracic pains and seemed to be without appreciable effect on the lesions. Although we failed in this particular therapy, it must be kept in mind that other workers have arrived at opposite conclusions with identical cases.

Pericardiac injections administered to three patients were easily tolerated, and seemed to slow down the evolution of the disease in two cases.

Three patients were treated by injections in the bladder:

A woman of 65 years who presented a bleeding and granulating carcinoma of a lateral aspect of the bladder received daily local injections of 200 μg. of actinomycin for a week. At the end of the first 24 hours the hematuria had ceased; during the following days the patient had cystitis, which subsided with sulfonamido therapy; later she received cobalt therapy.

A 68-year-old woman in serious condition, with a tumor of the right lateral aspect of the bladder accompanied by hematuria and cystitis, was improved by local injections of actinomycin to a total dose of 6000 μg. in five weeks. In her case intravenous treatment was impossible. Both hematuria and cystitis disappeared after the first few doses. The patient suffered no relapse and her condition improved; the volume of the carcinoma, however, remained unchanged.

The third patient was unimproved by the intravesical administration of 6000 μg. of actinomycin over a period of three weeks.

Not until September 1959 did we begin to apply local treatments to malignant skin lesions consequent to cancers of the breast or of the anus, or to metastases of various types of tumors. Compresses impregnated with 200 to 1000 μg. actinomycin C or D are applied *in loco* once a day. Although in some cases the treatment had to be discontinued because the patients were complaining of burning sensations, in others we obtained excellent results; the local applications seemed to promote the disinfection of the lesions and to reduce their size. In five cases of cancer of the breast actinomycin dressings were particularly helpful in checking the ulcerative tendency as well as the expansion of the lesions. Two patients who presented a recurrence of malignant lesions after amputation of the rectum were similarly improved by actinomycin dressings. One of them, however, complained of severe burning sensations and the treatment had to be discontinued.

We obtained results of comparable value in the treatment of ulcerative skin lesions secondary, in one case, to a carcinoma of the breast, in another, to a reticulosarcoma.

Treatment per os. It may seem surprising that we should have thought of giving actinomycin by mouth in view of its instability and its rapid neutralization by gastrointestinal secretions. Oral doses given experimentally to laboratory animals have been without antitumoral or toxic action. This has been explained by the fact that the peptic chains attached to the nucleus of actinomycin D are destroyed by the proteolytic enzymes of the gastric and duodenal juices.

We were therefore very much surprised at the positive results of oral administration to humans of actinomycins C and D. The cases observed are too numerous to be regarded as mere coincidences or ascribed to psychic factors. Furthermore, the appearance of secondary manifestations, such as cutaneous or digestive symptoms, or alopecia, were sufficient proofs that the actinomycin had actually been absorbed.

For a long time we hesitated to give actinomycin orally because of the risk of irritation of the gastrointestinal walls. However, no such symptom was reported. The solution given by mouth consists of 200 μg. actinomycin diluted in 5 ml. of bidistilled water.

The treatment *per os* may be prolonged for several weeks where other methods have failed. Several patients were able to tolerate doses of 20,000 to 30,000 μg. without untoward effects, but it does not seem that the daily doses can exceed those tolerated by intravenous injection. An attempt to raise the dose to 400 or 600 μg./day resulted in the appearance of intestinal symptoms within a few days.

A number of cases observed by us seem to bring indisputable proof of the action of actinomycin, administered orally, on malignant tumors.

A 59-year old man with cancer of the right upper lobe had been treated from October 5 to November 13 with daily doses of 200 μg. of actinomycin *per os*, when we decided to raise the daily dose to 400 μg. After a fortnight, however, the patient showed intestinal symptoms, and the treatment was interrupted from December 7 to 28 and then resumed with daily doses of 200 μg. Thus far the patient has received a total amount of 22,000 μg. The improvement in his case is striking, as shown by the retrocession of the radiological signs and bronchial decompression. The patient has gained a few pounds and gone back to work.

Several women who had previously undergone mammectomy followed by hormonotherapy and radiotherapy for carcinoma of the breast, came to us with multiple bone, pleural, ganglionic, and subcutaneous metastases. All were greatly improved by the oral administration of 10,000 to 30,000 μg. of actinomycin.

A patient with recurrent Hodgkin's disease with cervical localization, previously operated on, seems to have benefited from the absorption, in two months, of 11,000 μg. of actinomycins C and D *per os*. There was for some time a persistence of a subfebrile state, but this disappeared after the administration of daily doses of 500 mg. of phenylbutazone. At present the patient is in good general condition and the local induration that had developed after the operation is no longer present.

The possibility of administering actinomycin either orally or by local applications may be considered an important step. The medication, being better tolerated, may be administered over a longer period. Intravenous injections, on the other hand, will sooner or later cause sclerosis of the veins and, as a consequence, the treatment will have to be interrupted. In such cases, it is possible to resort to the administration of actinomycin *per os* until the veins have recovered their permeability.

The method of alternating series of intravenous and oral treatments has given most encouraging results. In this respect, we can report the case of a 27-year-old patient who, at the age of 23, had been operated on for an angioreticulosarcoma of the femoral blood vessels' sheath; following the operation, radiotherapy had been given. In May 1958 he began to cough and, several months later, round nodules were present in both lung fields. Injections of *N*-desacetylthiocolchicine hydrochloride brought temporary improvement, but after several months the nodules spread, until they finally extended over one half of both lung fields. Between September 15 and February 20, he received 22,600 μg. actinomycin in two series of intravenous injections of 8,000 μg. each, alternating with the oral administration of 6,600 μg. The improvement noted was considerable, and was evidenced by a retrocession of one third of the radiological pulmonary signs.

Combined Treatment

S. Farber[2] considers that actinomycin D has great potentiality with radiotherapy. The association in low doses of both treatments gives results at least equal to those obtained by a large administration of either actinomycin or radiotherapy. Tumors that resisted radiotherapy became sensitive to it after the addition of actinomycin. Several times we were able to lower the X-ray doses, lengthen the period of treatment, and prolong the patient's life. Moreover, the technique seemed to decrease the cutaneous and mucous reactions to radiotherapy.

For example, a woman of 30 years with a subpleural mesothelioma was treated by lobectomy. Eighteen months later she developed a syndrome of mediastinal compression and received a first dose of 3500 μg. actinomycin, then 8000 r in 40 sessions, then a new dose of 1000 μg. actinomycin. After this the radiological signs almost disappeared. The patient survived for about a year in normal condition, but died in the course of a few days from sudden paraplegia probably resulting from a spinal metastasis.

A man of 53 years presenting a reticulosarcoma with important bilateral axillar and inguinal adenopathy received 10,000 μg. actinomycin in February 1954. This treatment resulted in an important decrease in ganglionar volume. It was followed by small doses of X ray (about 3700), then by a second treatment of 7200 μg. actinomycin from May to June 1954. The adenopathy disappeared and the patient was able to lead a normal life until November 1955, when leukosis appeared. The leukosis regressed after another series of actinomycin doses, associated first with prednisone, then with 6-mercaptopurine. The patient died in 1957.

A 56-year-old woman presented in 1949 a small mass in the right breast. The mass was surgically removed, after which she underwent numerous séances of deep X rays. Eight years later, in January 1957, she had a relapse but refused to undergo another operation. She came to us at the beginning of January 1960 with an ulcerative granulating carcinoma involving one third of the hemithorax wall. The chest wall was destroyed down to the ribs and was the seat of a suppurative fetid mass. With little optimism we decided to apply actinomycin dressings, giving a daily dose of 1 mg. The result was amazing. Within two days the infection had regressed. The lymphangitis, which had been extending to the left upper limb, disappeared, the fetor decreased, the wound cleared up, and part of the malignant proliferations were eliminated. In the presence of such unexpected results we gave her doses of 200 μg. of actinomycin every other day. After five doses, however, we had to interrupt the treatment because the patient complained of nausea. We decided then to add radiotherapy to the treatment, and she was given 2250 r during three weeks. The improvement was remarkable: the neoplastic proliferations disappeared from the upper part of the wound, scar tissue formed, the ulceration diminished in size and the granulations disappeared almost completely. Obviously such excellent results are due to the association of actinomycin and radiotherapy.

Actinomycin Treatment Associated with Cytostatic Compounds

Most cytostatic compounds present a grave objection in that they injure severely the bone marrow. They may provoke an intense leukopenia and, after more or less time, anemia.

Such effects obviously obliged us to interrupt treatment and, during these intervals, actinomycin, which has almost no effect on bone marrow, allowed us to continue using cytostatic compounds. We obtained a certain number of satisfying results. For example, a woman of 53 years, who had undergone ablation of the left breast with ganglionar curetting of the armpit and total hysterectomy, some time later was given 60 treatments of radiotherapy. A year later, she exhibited recurrence of the lesions, which became ulcerated. From September 1959 she received 360 mg. of Thio-TEPA and daily dressings of actinomycin. By the end of December 1959 the cutaneous nodules had decreased in number and size and the ulceration had ceased.

In the middle of January 1960 this patient also took daily oral doses of 200 μg. actinomycin C for a fortnight. This treatment was interrupted for a week and then resumed. Actinomycin dressings were applied for two months, stopped for 15 days, and reapplied for a new period of two months. After that time the patient presented a slight cutaneous eruption that disappeared a few days after the treatment had been discontinued. Her condition is now considerably better; the skin lesions have retroceded by almost one third. The treatment is being continued.

A 64-year-old woman who, 20 years before, had undergone a right mammectomy for carcinoma of the breast, presented an identical tumor in the left breast. Amputation of the breast and curettage of the axillary ganglions were soon followed by local recurrence and right neoplastic pleuritis. The evolution was retarded by various treatments such as radiotherapy, hormonotherapy, ethyleneimine quinone. Several series of intrapleural injections of actinomycin improved the pleuritis. The skin lesions, which had degenerated into a scirrhous carcinoma with nodular and ulcerative lesions, were markedly improved by local actinomycin dressings, which prevented their further extension.

A 50-year-old woman was operated on in February 1959 for a carcinoma of the right breast, following which she had radiotherapy. In December 1959 a metastasis appeared on the 10th vertebra. Radicular pain obliged her to remain in bed. She was considerably improved by alternated treatments of 300 mg. Thio-TEPA, and 6000 μg. actinomycin D, and became able to leave her bed.

Side Effects

The side effects observed after the administration of actinomycins C and D are very similar to the side effects of radiotherapy. Both actinomycins have identical degrees of toxicity, although actinomycin D usually seems to be better tolerated.

The side effects consist chiefly in irritation of the gastrointestinal mucous membranes, accompanied by nausea, anorexia, diarrhea, and ulcerative or aphthous stomatitis. They may be associated with lingual exulceration and cheilitis that interfere with normal alimentation for several days.

Sometimes patients also present intestinal, vaginal, or oral mycosis that regresses easily after interruption of the treatment.

We have observed several cutaneous eruptions of erythematous or eczematous types, as well as partial or general alopecia.

It is possible to prevent these side effects by taking certain measures, for example, by keeping the doses between 200 and 400 μg.

Furthermore, it is most important to detect the first symptoms of intolerance as soon as possible and to interrupt the treatment at once and for several days.

We think it necessary, before beginning any treatment, to determine whether patients are suffering from dental infection and to treat such infection first. It also seems best to associate a polyvitamin therapy with actinomycin.

However, such measures are not always sufficient to prevent toxic accidents. Some patients present phenomena of intolerance after intravenous injections even of small doses. We recently saw a patient who presented an erythrodermia—which, however, easily regressed—that appeared after only one dose of 200 μg. of actinomycin.

We must say that most of the accidents, even when important, are rather short-lived and leave no after-effects. Alopecia, for example, is always temporary.

It is also important to say that side effects usually are seen after intravenous injections and, although actinomycin orally administered may bring some, its effects never appear as promptly and never have the same importance.

On the other hand, we have never encountered accidents when actinomycin C was injected into the serous membrane. Sometimes we have noted pains after intrapleural injections, or temporary inflammation after intraperitoneal injections. They probably resulted from the surging back of a certain amount of the drug into the subcutaneous cellular tissue.

We turn now to a point that must be stressed: the great rarity of toxic blood accidents caused by actinomycin.

Janbon, Bertrand, and Carli[3] reported the cases of two patients, treated with high doses of actinomycin C, who died from agranulocytosis. In both cases, however, the daily dose of 1000 μg. (which at the present stage of our experience we deem too high) had been reached and were maintained even after the appearance of intolerance. We think it extremely important to interrupt the treatment temporarily as soon as the first signs appear.

We observed a patient who, after administration of 7000 μg. of actinomycin, presented purpuric and thrombocytopenic symptoms, which regressed as soon as treatment was interrupted and appeared again when the treatment was resumed. A month and one half after treatment had been discontinued the patient presented hemorrhagic symptoms with fever, soon followed by fatal generalized cancer. In this case it was difficult to discern the effects of the actinomycin.

Among the patients we treated we observed only a few and unimportant changes in the blood formula and bone marrow and, actually, routine tests are made at long intervals.

Summary

From the cases we have observed, it appears clear that actinomycins C and D bring only temporary and partial improvements in hemopathic conditions and malignant tumors. Nevertheless, actinomycin is one of the most effective anticancerous substances presently known. In low doses it ensures notable regression of transplanted carcinomas in mice and of varied human tumors inoculated in hamsters. Although the mechanism of its activity is still

unknown, it seems to have a direct action on tumoral cells without the intervention of metabolic modifications.

Its action in the human being is more obscure, and is only temporary. However, this is true of all other anticancerous substances or therapeutics.

Actinomycin has noticeable effects on the lymphatic system, and for this reason gives better results in chronic leukemia and Hodgkin's disease than in malignant tumors.

We are inclined to be more optimistic in our conclusions than Farber, who states that "with the exception of the effect on the Wilms' tumor metastases, actinomycin D, when used alone, did not produce sufficiently important therapeutic effects, with regularity, to earn for itself a place in the therapeutic armamentarium against cancer."

The beneficial action of actinomycins C and D in association with radiotherapy is indisputable. They allow prolongation of treatment and the remissions of various forms of cancer have been sufficient in number and duration for us to acknowledge their intrinsic therapeutic value. Unquestionably, they are more than mere contributions to radiotherapy. Moreover, their relative lack of toxicity to the bone marrow makes it possible to use them alternately or concurrently with other more toxic cytostatic compounds.

The local and oral administration of this medication represents a further and important development in actinomycin therapy.

The mechanism of actinomycin's action deserves to be investigated further. A precise knowledge of the structure of the compound would enable us to find a substance of similar origin that would combine a lower degree of toxicity with an equal or even higher anticancerous activity.

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IN VITRO STUDIES WITH HELA CELL LINES SENSITIVE AND RESISTANT TO ACTINOMYCIN D

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There has been increasing interest in the retardation of tumor growth by certain antibiotics.[1-7] Actinomycin D is one of several antibiotic substances that have caused regression of both experimental and some human tumors. Very little is known, however, of the mechanism of action of the actinomycins in mammalian or bacterial cells. Foley showed that in certain bacteria the growth-inhibiting effects of actinomycin D could be reversed by pantothenate and a number of other compounds.[8] On the other hand, the toxic effects of this drug in *Bacillus subtilis* 6051 could not be prevented by the addition of pantothenate or a variety of other compounds. Slotnick found that actinomycin D depressed the synthesis of protein and ribonucleic acid (RNA) in *B. subtilis*, while deoxyribonucleic acid (DNA) appeared unaffected.[9-11]

Craveri and Veronesi[12] reported that actinomycin M (one of a series of actinomycins which varies in the structure of its peptide chain) affected a variety of mammalian, avian, and plant cell types studied *in vivo* and *in vitro*. They reported that the drug caused (1) a decrease in the mitotic index, (2) inhibition at prophase when administered in high doses, (3) cytoplasmic and nuclear vaculation, nuclear hypertrophy, pyknosis, karyorrhexis, and karyolysis in proliferating cells, but had little effect on nonproliferating cells. The germinative tissue of rabbit testis was not affected. They concluded from their observations that actinomycin M was a "mitotic poison" acting mainly on the "chromosomes" in preprophase and could be classified as a "radiomimetic."

Actinomycin D inhibits the growth of cells of established lines as well as of cells in newly explanted cultures, and is effective at very low concentrations.[13-15]

Cobb and Walker[4] reported that actinomycin D produced a range of nonspecific cytotoxic effects, including granulation, pycnosis and, sometimes, reduction in the size of the nucleoli. Only eight of nineteen primary explants of neoplastic tissue showed cytotoxic changes when exposed to very low concentrations of actinomycin D.

The present studies were undertaken to learn more about the effects of actinomycin D on HeLa cells that were sensitive to the antibiotic, and to report the development and characteristics of HeLa cells that are resistant to the drug.

Materials and Methods

The HeLa line sensitive to actinomycin D (HeLa-S) and a line made resistant (HeLa-R) were both grown in a medium composed of 1 part calf serum and 2 parts medium 199. Four hundred units of penicillin, 400 U./ml., and streptomycin, 80 μg./ml., were routinely added to the medium.

Stock actinomycin D was prepared by dissolving 10 mg. in 2 ml. of absolute ethanol and diluting to 100 ml. with distilled water. This solution was sterilized by filtration through a 02 Selas candle.

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Bright field microscope observations were made of HeLa cells growing in D 35 Carrel flasks. For more detailed phase microscope observations, cells were grown on microslides or on 40 by 45 mm. No. 1 cover slips and Gey cover slip preparations were made.

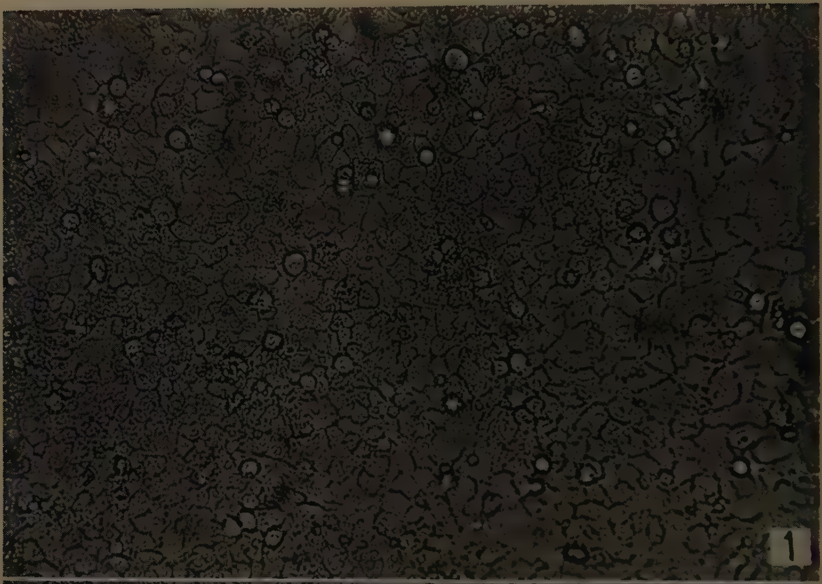
Cytological and cytochemical studies were done on HeLa cells grown on standard 3- by 1-inch microslides in square French bottles. After growing for varying periods in 10 cc. of medium containing actinomycin D, the slides were washed with Hank's solution and fixed and stained by the following procedures. Cells fixed in Lillie's fixative were stained with hematoxylin and eosin or toluidine blue. The methyl green-pyronin technique of Trevan and Sharrock[16] was used as an indicator of RNA after fixation in 10 per cent neutral formalin. The DNA was determined in individual HeLa cells by Feulgen cytophotometry employing an instrument previously described.[17]

Results

Development of the HeLa-R line. HeLa-S cells (FIGURE 1) were grown in D 35 Carrel flasks until a continuous sheet of cells covered the floor of the flasks. The cells were then exposed to 0.1 μg./ml. of antibiotic for 48 hours. After 48 hours approximately 75 per cent of the cells were so badly damaged that they were loosened from the glass surface. The cells that remained attached to the glass were vacuolated and exhibited very granular cytoplasm. All of the surviving cells contained nucleoli that were extremely small compared to the large prominent nucleoli of control HeLa cultures. The surviving cells were washed 3 times and then incubated in control medium. After 5 to 6 days they had sufficiently recovered to resume multiplications and in 12 to 14 days had again proliferated to form sheets of epithelial cells. The cultures were again exposed to actinomycin D and the same procedure repeated. After 20 weeks of intermittent exposure and recovery, a cell line (HeLa-R) was developed that could actively multiply in 0.1 μg./ml. of actinomycin D at the same rate as the HeLa-S cells could in control medium.

Partial resistance was apparent in the HeLa cells by the 16th week of intermittent exposure to actinomycin D. These HeLa cells were characterized by longer survival (12 to 14 days) in the presence of 0.1 μg./ml. of the antibiotic. The nucleoli in these cultures were not altered as they were in the sensitive cells and a reduced rate of mitosis persisted in cultures for 6 to 7 days. Recent studies have shown that loss of resistance in HeLa-R cells growing in normal medium also occurs gradually. After 12 subcultures at weekly intervals, the HeLa-R line cells became increasingly sensitive to the toxic effects of the antibiotic and by the 28th week were as sensitive as HeLa-S cells.

Cells of the HeLa-R line (FIGURE 2) are distinguished by their dense small nucleoli, usually one to three in number. The nucleoli of the resistant cells were intensely stained with toluidine blue, eosin, and fast green, and the cytoplasm showed increased basophilia when compared with HeLa-S cells. Although both cell lines grow as sheets of epithelium, the resistant line is further differentiated (after more than one year of culture in media containing actino-



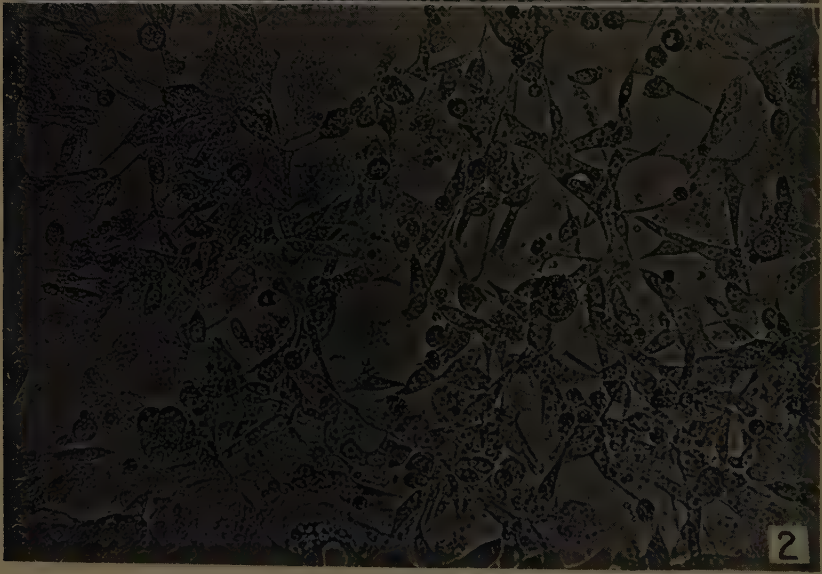


FIGURE 1. Continuous sheet of living HeLa-S cells in D 35 Carrel flask. Bright field. Photographed with Galileo inverted microscope. ×110.

FIGURE 2. HeLa-R cells exposed to 1.0 μg./ml. actinomycin D for 12 days (medium changed 3 times weekly). Note the conversion of what was previously a sheet of epithelium-like cells into fibroblastlike cells in the presence of the antibiotic. Photographed with Galileo inverted microscope. ×110.

mycin D) by the more uniform size of its cells and also by the presence of small numbers of multinucleated giant cells, which are observed only rarely in cultures of the sensitive HeLa line.

Mitosis. When HeLa-S cells were exposed to 1 μg./ml. of the antibiotic during the log phase of growth, many abnormal prophases accumulated in cultures (FIGURE 3). The chromosomes were shorter and more thickened than those of controls at the same stage of division, and the nuclei were larger than those of control cells carrying the same chromosome number. The nucleoli were smaller than those in the control cultures at the same stage of prophase (FIGURES 3 and 4). The other cells in these cultures were in interphase, except for a small number of cells with pyknotic nuclei.

Cells in interphase: response of HeLa-S cells. Sensitive HeLa cell exposed to 1 μg./ml. actinomycin D showed the following changes. The nuclei were larger than those in cells of control cultures (FIGURES 5 and 6). In stained preparations the nucleoli either appeared as small condensed masses in a clear hyaline court or were not stained at all by hematoxylin or toluidine blue.

When the nucleoli were stained with eosin, fast green, or pyronin (which also combines with protein as well as RNA) they were only lightly stained. Very few nucleoli were unaltered, the majority being markedly changed in morphology and in their staining characteristics.

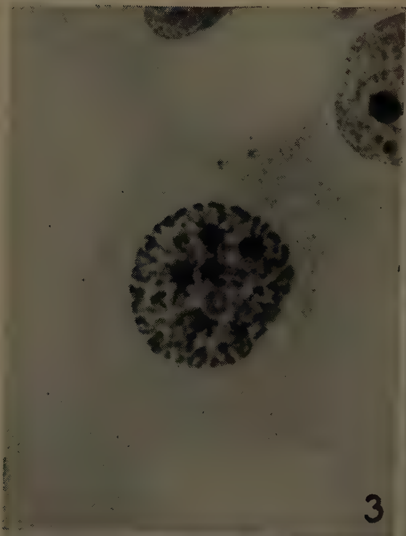
These findings were corroborated by electron microscope examination of osmium-fixed cultures. The dense, uniformly osmiophilic nucleoli of HeLa-S cells were converted to nucleoli possessing a hyaline interior surrounded by a peripheral ring of darkly stained granules, after exposure to actinomycin D. Budding and fragmentation of nucleoli was another consistent feature of drug-treated HeLa-S cells.

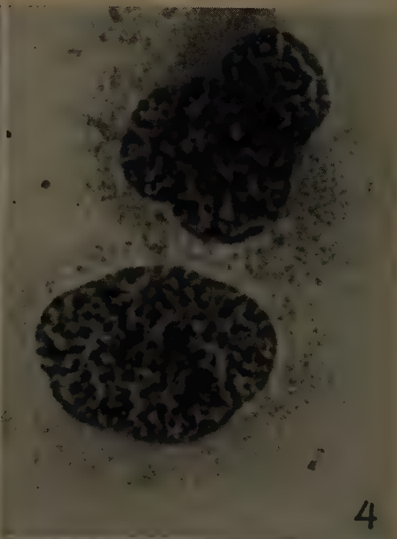
In some instances strands of nucleolar material radiated from a central condensed basophilic mass (FIGURE 7). These strands were attached to the nucleolus-associated chromatin, and may represent points of attachment of the filamentous nucleoli to the associated DNA. In other nuclei, the nucleolar area was represented by a hyaline region surrounded by nucleolus-associated DNA (FIGURE 7).

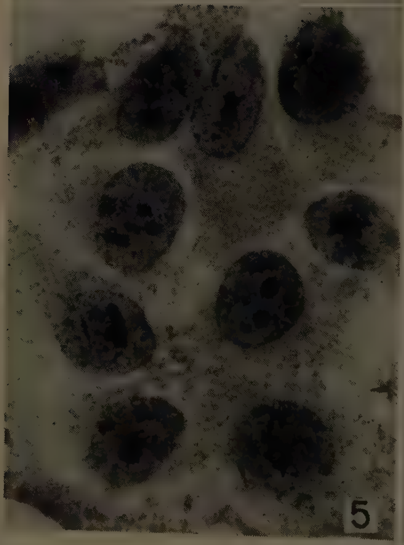
The nucleolus-associated DNA appeared to increase in treated cells, and in some cells it formed dense condensations (FIGURE 6). Similar nucleolar changes were found in interphase cells, even when the concentration of actinomycin D was reduced from 1 to 0.01 μg./ml.; for example, five to seven days after exposure to the latter concentration cytotoxic changes began to develop in HeLa-S cells.

Cells exposed to actinomycin D frequently contained large cytoplasmic vacuoles, which were sometimes present in cells for many weeks after recovery from exposure to the antibiotic. During the recovery period divisions could be seen in cells even though these large vacuoles persisted in the cytoplasm.

Although no attempt was made to quantitate the RNA and protein content of stained HeLa-S cells exposed to actinomycin D, qualitative differences were readily apparent by the intensity of the staining reactions in both treated and control cells. The cytoplasm of actinomycin D-treated cells in interphase stained less intensely with pyronin, fast green, and eosin. Preparations stained







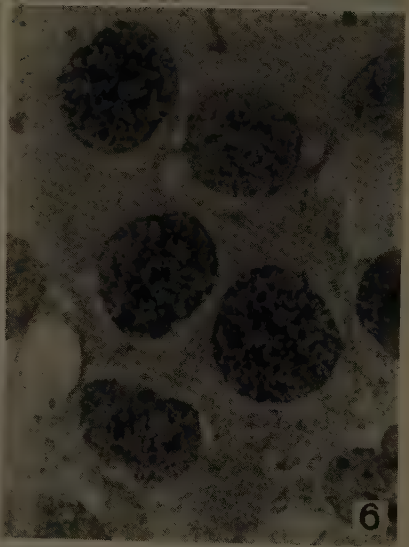


FIGURE 3. HeLa-S cell in control growth medium. Normal prophase. Note the filamentous chromosomes and densely stained nucleoli. Lillie. Hematoxylin and eosin. ×900.

FIGURE 4. HeLa-S cells treated for 24 hours with 1 μg./ml. actinomycin D in medium. Note the short thickened chromosomes of two cells in prophase. The nucleoli are not as prominent as in the previous figure. The nuclei of these and other cells in prophase are larger than those of cells in normal growth media. Lillie. Hematoxylin and eosin. ×900.

FIGURE 5. HeLa-S interphase cells in control growth medium. Note the prominent nucleoli in these cells. Carnoy. Feulgen and fast green. ×780.

FIGURE 6. HeLa S culture treated with 1 μg./ml. actinomycin D. The nucleolus-associated DNA has become dense and has accumulated in clumps around the nucleoli of some cells. Note the increased size of the nuclei and the less intensely stained cytoplasm. Carnoy. Feulgen and fast green. ×780.

with toluidine blue and those stained with hematoxylin also showed decreased cytoplasmic basophilia. A progressive loss of staining intensity was observed as the cultures were exposed for longer time periods. These observations indicated that cytoplasmic RNA and protein, as well as nucleolar RNA and protein, were either decreased in amount or were in some way altered by the antibiotic.

Phase microscope observations showed that the earliest morphologic change occurred in the nucleoli of actinomycin D-treated cultures (FIGURES 8 and 9). Even in cultures treated for long periods at 0.01 to 0.001 μg./ml. concentrations of actinomycin D, the most significant morphologic change in the cells was in the nucleoli. The mitochondria in cells treated with the antibiotic appeared normal in morphology, although many of these cells contained nuclei with no visible nucleoli.

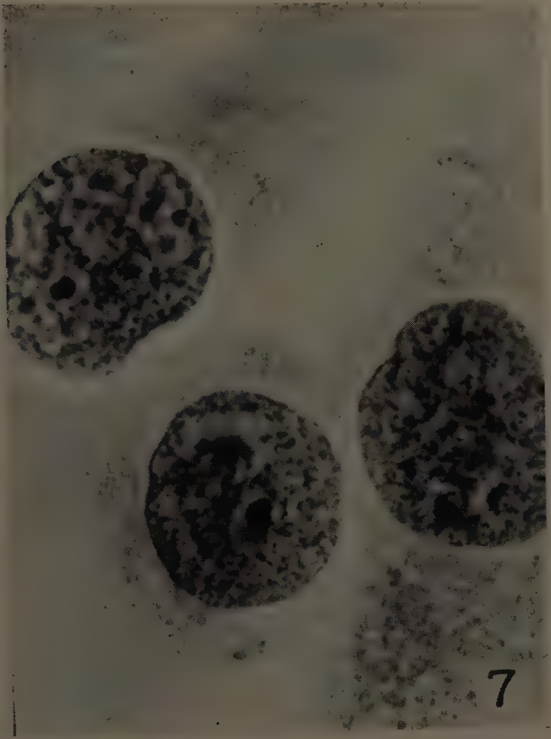
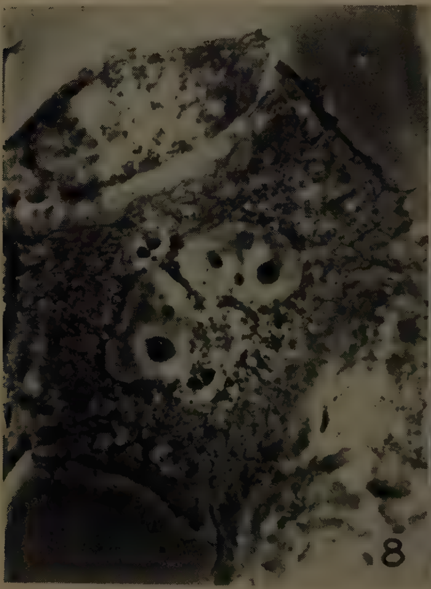


FIGURE 7. HeLa-S cells exposed for 24 hours to 1 μg./ml. actinomycin D, Note the enlarged nuclei and marked change in structure of the nucleoli. Some nucleoli are poorly stained, and in others strands of basophilic material radiated from the condensed nucleoli and were attached to the nucleolus-associated DNA. Lillie. Hematoxylin and eosin. ×1350.

An unusual change in morphology occurred when resistant cells were exposed to even higher concentrations (1 to 3 μg./ml.) of actinomycin D. Although the resistant cells survived for long periods in media containing these concentrations, there was a gradual decrease in the number of mitoses over a period of 8 to 10 days and a conversion of the epithelial cells into cells with a fibroblastlike morphology (FIGURES 1 and 2). This change in morphology is similar to the degenerative changes seen in cells deficient in certain essential amino acids or vitamins. Detailed electron-microscope studies of this phenomenon also are in progress.

DNA determinations in individual cells of HeLa-S. The DNA was determined by Feulgen cytophotometry in individual HeLa-S nuclei exposed to 1 μg./ml. of actinomycin D for 24 hours. The HeLa-S cells in normal media had a distribution histogram characteristic of a heteroploid population of cells having different basic amounts of DNA and actively synthesizing new DNA (FIGURE 10). Upon exposure to 1 μg./ml. of actinomycin D for 24 hours there was an increase of 12.5 per cent in the mean DNA content. This quantitative increase, measured by Feulgen cytophotometry, correlated closely with our morphologic observations. Thus it appears that HeLa-S cells were capable of synthesizing DNA in the presence of actinomycin D, but were not able to divide. Additional evidence in support of this view was obtained when HeLa-S cells were labeled with tritiated thymidine and examined 24 hours later. Cells growing in media containing actinomycin D were more heavily labeled than cells growing in normal growth medium.



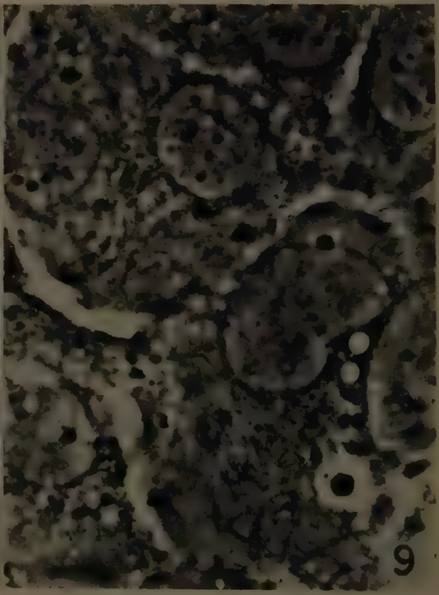


FIGURE 8. HeLa-S cells in normal medium. Compare nucleoli with those in FIGURE 6. Leitz-positive phase contrast. ×700.

FIGURE 9. HeLa-S cells exposed to 0.01 μg./ml. actinomycin D for 7 days. Nucleoli appear as small dense spheres. In some cells it was difficult to find them. The mitochondria are like those of cells in control medium. Leitz-positive phase contrast. ×780.

Biochemical studies. Preliminary investigations showed that, at concentrations of actinomycin D ranging between 0.10 and 0.30 μg./ml., marked changes in the cytology and cytochemistry of cells could be produced within 48 hours. At these concentrations it was still possible to obtain sufficient numbers of viable cells for chemical determinations. Cells were exposed to the antibiotic near the end of the rapid phase of growth, and in this way large samples were obtained for the determinations of DNA, RNA, and protein.

No noticeable effect was produced by actinomycin D (0.25 μg./ml.) on the DNA, RNA, or protein content of HeLa-S cells after 24 hours of exposure. After 48 hours, however, there was a significant decrease in RNA concentration. The ratio of total protein to RNA (which was used as the standard of comparison) had increased from 35 at 24 hours to 90 at 48 hours. This represented an almost twofold decrease in RNA. There was also a decrease in total protein content. These preliminary biochemical studies of cells sensitive and resistant to actinomycin D are being extended.

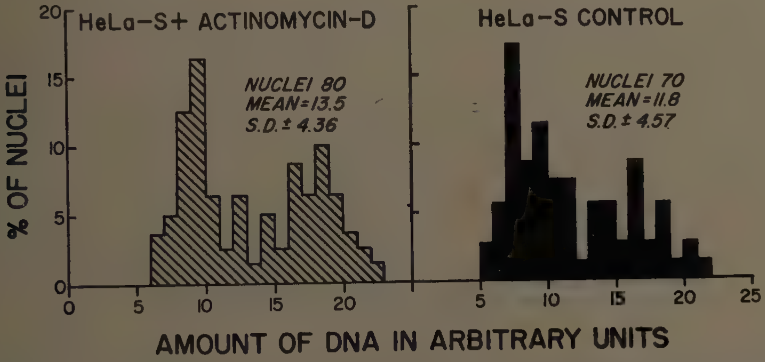


FIGURE 10. Histograms, determined by Feulgen cytophotometry, of the DNA of individual HeLa-S cells exposed to 1 μg./ml. actinomycin D for 24 hours and corresponding control.

Discussion

Cytological studies of living and fixed HeLa cells showed that the nucleolus was the first structure to be altered when cells were exposed to actinomycin D. Our staining procedures indicated that there was a decrease in RNA and protein in the nucleoli of cells exposed to the antibiotic, and this observation was confirmed by direct chemical determinations of these constituents. RNA in HeLa-S cells was markedly reduced in cultures exposed to actinomycin D, and the protein concentration also was decreased although to a lesser extent.

The role played by the nucleolus in cell multiplication and function is incompletely understood. It is known that numerous factors are able to affect the size and shape of the nucleolus;[18-20] for example, starvation or feeding of protein-free diets may cause reduction in the nucleolar size in rat liver. Firket *et al.*[18] have described the effects of ribonuclease (RNAse) on chick myoblasts and fibroblasts growing in tissue culture. The effects parallel the changes observed in HeLa cells exposed to actinomycin D. These investigators were able to reverse the effects of RNAse with animal or yeast RNA. Although the changes in HeLa cells treated with actinomycin D resemble those of chick cells treated with RNAse, we could not reverse the effect of actinomycin D with yeast or animal RNA. (Attempts to produce the changes described by Firket *et al.* with 2X crystallized salt-free RNAse, added to living HeLa cells grown in a variety of media, were unsuccessful at concentrations up to 4 mg./ml.)

The morphologic and cytotoxic studies reported by Craveri and Veronesi,[12] Eagle and Foley,[13,14] and others, and our own observations suggest that the various actinomycins have a common mechanism of action and probably affect susceptible cells in the same way. We do not believe, however, that this cytotoxic antibiotic should be classified as a "radiomimetic" or alkylatinglike agent. Actinomycin D does not produce the chromosome breakage and over-all cell swelling typical of alkylating agents of X rays.[21] One of the early effects of X irradiation is the inhibition of DNA synthesis while RNA and protein synthesis continue.[22] Actinomycin, on the other hand, inhibits RNA and protein synthesis.

Nitrogen mustard and mitomycin C also have been added to cultures of HeLa-S and HeLa-R cells. Neither of these compounds produced the specific alterations in HeLa-S cells that were caused by actinomycin D. The morphologic alterations produced by high concentrations of nitrogen mustard did, however, resemble the effect of X irradiation (unpublished observations).

Certain bacteria, a number of transplantable animal tumors, and some human tumors growing *in vivo* are not affected by actinomycin D.[5,6,23] Katz and Pienta[23] reported that there are certain soil organisms that can degrade actinomycin D. Resistant tumors may have a similar degrading enzyme or, by some mechanism of enzyme induction, cells previously susceptible to the agent may now be able to inactivate the antibiotic. Another possibility is that resistant cells can develop alternate pathways for the synthesis of RNA and protein.

In order to evaluate better the possible mechanisms and sites of action of potential antitumor compounds, one should utilize biochemical and histochemical techniques in close conjunction with phase and electron microscopy.[24] This somewhat more tedious approach would have avoided, for example, the misclassification of actinomycins as "radiomimetics." Our studies indicate that actinomycin D has a specific effect on the morphology of the nucleolus; this should make it an important and useful tool for studying the function of the nucleolus in both normal and malignant cells.

Acknowledgments

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SUMMARY: THE ACTINOMYCINS AND THEIR IMPORTANCE

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Just 20 years have passed since the isolation of actinomycin, the first crystalline antibiotic obtained from the culture of an actinomycete. The antibiotic proved to be too toxic to justify any attempt to use it therapeutically in the treatment of infectious diseases of animals and man. However, this very toxicity appears to be largely responsible for its more recently discovered antineoplastic properties. Thus actinomycin combines in its molecule the capacity to inhibit the growth of microbes, thereby justifying its designation as an antibiotic, and the capacity to inhibit the growth of certain abnormal cells of the host, namely, tumor cells, thereby justifying its designation as an antineoplastic agent. It is this last property that has led to the publication of this monograph.

The antibiotic potentialities recognized in actinomycin led to extensive studies of actinomycetes as producers of such compounds, and the discovery of numerous other antibiotics that proved to be of great therapeutic significance soon followed. Beginning with streptomycin, one need mention only the following: chloramphenicol, the tetracyclines, neomycin, nystatin, the erythromycins, and numerous others. Unfortunately, the progress made in finding new anticancer agents in cultures of actinomycetes was very limited.

Whereas one hardly thinks at present of actinomycin as a potential chemotherapeutic agent for bacterial or fungal infections, its antineoplastic properties have come to the fore only recently. The reason for this belated recognition may be due, partly at least, to the lack of better agents, agents that would be less toxic or more effective cr both. The great progress in the isolation of antibiotics encouraged the hope that further searches for antiviral and antineoplastic agents would lead to entirely new substances. Unfortunately, the lack of progress has thus far been very discouraging, and has led, therefore, to a re-examination of the potentialities of actinomycin in this direction.

The papers comprising this publication establish several important facts and raise several important questions that may be summarized briefly as follows:

It has been amply established that the biosynthesis of different forms of actinomycin can be controlled by feeding the culture certain special amino acids, such as sarcosine, and by controlling conditions of growth, notably, aeration and reaction. The announcement made in these pages of the chemical synthesis of actinomycin raises further the great potentiality of producing numerous modifications in the structure of the actinomycin molecule. One can hope that certain forms of actinomycin will be found that will prove to be less toxic to animals. Although such compounds have actually been isolated, at least some of them have also proved to be less active. The apparent parallelism between antineoplastic activity and toxicity has been suggested, although there is no doubt that some preparations at least can be less toxic and more active. It has also been demonstrated that different neoplasms are sensitive in different degrees to the various actinomycins. Crude filtrates may actually be more active than isolated and purified compounds.

The above facts raise the following questions: Should one look to further studies of the actinomycins for more, rather than less, toxic preparations, hoping thereby that they will also be more active? Should one use for clinical purposes more complex, rather than purified, preparations, thus expecting a wider range of activity? Or should one attempt to use mixtures of purified components, thus broadening the spectrum of potential activity? These questions require further study and elucidation.

Let us hope that this monograph will have served as a milestone, not only in the development of our knowledge of the nature, activities, and utilization of actinomycin in the treatment of both experimental and clinical tumors, but also in the development of our ideas concerning the search for microbial products that possess antineoplastic properties.

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